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SPONTANEOUS MUTATION AT THE *R* LOCUS IN MAIZE

III. GENETIC MODIFICATION OF MUTATION RATE

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Among R^r stocks tested for frequency of seed-color mutation, the "Cornell stock" showed the highest mutation rate, 18.2×10^{4} (Stadler, 1948). Extracted stocks homozygous for R^r : Cornell, derived from crosses of the Cornell stock with other stocks, showed that this allele is not unusually mutable, as compared with other R^r alleles. Its high mutation rate in the Cornell stock is therefore due to the presence in this stock of modifying genes favorable to its mutation. The present paper is concerned with an attempt to identify genes modifying the mutation rate of R^r : Cornell.

A multiple linkage tester stock, *B a su pr Y-Pl J wx* R^g, was crossed with the Cornell stock (*b A Su Pr y-pl j Wx* R^r). The marker genes listed are located respectively on chromosomes 2, 3, 4, 5, 6, 8, 9, and 10. The F_1 progeny, tested for seed-color mutation rate of *R*, yielded no mutations among the 24,339 female gametes tested. Since the F_1 plants tested for mutation were R^r R^g in constitution, about half of the gametes tested were R^r : Cornell gametes. In a population of 12,170 gametes, in the Cornell stock, the expected yield of seed-color mutations would be 22. Thus the total effect of the genes of the tester stock which differed from those of the Cornell stock, as shown in plants which were heterozygous for all these genes, was a pronounced reduction in the frequency of mutation of the R^r: Cornell allele.

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[1] Cooperative investigations of the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, U S. Department of Agriculture, and Department of Field Crops, University of Missouri. Missouri Agricultural Experiment Station Journal Series No 1139.

The part played in this effect by each of the chromosome-segments bearing a marker gene (except for the segment marked by *a*) may be estimated in the progeny produced by backcrossing the F_1 to the Cornell stock. For example, considering only the segment marked by *B*, half of the plants of the backcross progeny will be *B b* and half *b b*. Now if a factor *M*, reducing the mutation frequency of R^r, is located in the segment marked by *B*, it will tend to be present in the backcross progeny in plants of the *B b* group, and to be absent in plants of the *b b* group. It will thus tend to result in a lower frequency of mutation in the former group than in the latter. Plants in which *M* is present will in all cases be heterozygous for this factor, for all plants of the backcross progeny will carry also the contrasting allele from the Cornell stock. Thus the backcross can identify only factors derived from the tester stock which are dominant to the corresponding factors present in the Cornell stock. Dominant factors increasing mutation frequency, derived from the tester stock, would be identified similarly, by increased rather than decreased mutation-frequency in the group identified by the marker gene (the *B b* group in the example given). The term "dominant" is here used in the broadest sense, to apply to any allelic relation in which the heterozygote *B b* differs recognizably from the homozygote *b b*. It is not implied that the effect of *B b* is more like that of *B B* than that of *b b*, for in this experiment it is not feasible to determine the homozygous effect of the mutation modifier derived from the tester stock.

The same backcross progeny may be classified similarly for the other segregating marker-genes, for evidence of mutation modifiers associated with the chromosome segments marked. For each of the dominant plant-character genes of the tester stock, *B, Pl* and *J*, the two classes of backcross individuals concerned are readily identified by inspection of the backcross plants. For the marker genes of which the tester stock carries a recessive allele (*i.e., su, pr, y, wx,* R^g), the two classes of backcross individuals are the homozygous dominant and the heterozygote (*e.g., Su Su* and *Su su*). Since these genes are identified by seed or seedling characters, the two classes may be made distinguishable by using as the pollen stock, in the detasseled plot in which mutation frequency is determined, a stock recessive or heterozygous at each of these loci. The pollen stock used was r^g *Su su pr y wx.* It was therefore possible, by inspection of the ears, to classify the backcross plants for *su, pr, Y,* and *wx.* Seedling cultures, grown from 20-seed samples from each plant, permitted the further classification of the backcross plants as to R^g ($R^r R^r$ vs. $R^r R^g$). Classification for *a* could not be accomplished in this way, since its phenotypic effect conflicts with that of *r.* The addition of *a a* or *A a* to the pollen stock used in the detasseled plot would have made it impossible to detect the seed-color mutations of *R.*

Each of the plants of the backcross progeny could thus be classified as to the presence or absence of a chromosome segment from the tester stock, in the case of each of seven genetically marked segments, one of which was marked by the two genes *Y* and *Pl.* The data on mutation frequency in the 224 plants of the backcross progeny could then be classified successively as to each of the eight marker-genes, to determine any differences in mutation rate associated with the presence of these chromosome segments.

Obviously, a factor modifying the mutation rate will be detectable in the backcross progeny only if it has a pronounced effect upon mutation frequency. The effect must be large enough to produce a significant difference in mutation rate in the contrasted classes, in the face of the high sampling error inevitably associated with mutation rates. Consider, for example, a backcross progeny yielding 30 mutations, the number actually identified in the backcross progeny used in this study. Suppose that one-half of the gametes tested are from *B b* plants, and one-half from *b b* plants. How large a difference in mutation frequency is required for reasonable assurance that the difference observed is not due to sampling fluctuations? Assuming that a mutation-modifier closely linked with *B* actually doubles the mutation rate, and that twice as many mutations are identified in *B b* plants as in *b b* plants, the difference observed would be statistically insignificant. The observed result in this population would be 20 mutations in one class versus 10 in the other. A deviation from equality as large as this would occur by chance, if mutation were equally probable in the two classes, with a probability slightly greater than 0.05, and the result would therefore be considered statistically insignificant by conventional standards.

The effect of crossing over between the mutation-modifying gene and the marker gene is to reduce the detectable effect, and thus to reduce the probability of detection. For example, suppose that a modifier *M* results on the average in a ten-fold increase in mutation rate, and suppose that the modifier is linked with *B* with 20 per cent. crossing over. The comparison in mutation rate is made between *B b* plants and *b b* plants. Assuming for illustration uniform progenies of 100 seeds per plant and a uniform mutation rate of 0.1 per cent., except for the action of this modifier, the result would be as follows:

	B b plants			*b b* plants		
	No.	Genotype	Mut Frequency	No.	Genotype	Mut. Frequency
Non-Crossover	80	B M/b m	80/ 8,000	80	b m/b m	8/ 8.000
Crossover	20	B m/b m	2/ 2,000	20	b M/b m	20/ 2,000
Total			82/10.000			28/10.000

Thus the 10-fold increase in mutation rate, which would be evident if there were no crossing over between the modifier and the marker gene, is reduced to less than a 3-fold increase, as shown by the comparison of *B b* and *b b* plants. The minimal increase required to make a modifying factor detectable in the experiment applies of course to its net effect after this reduction has been made.

Accordingly, it may be expected that the comparison of mutation rates in the contrasted classes of the backcross progeny will identify only a small proportion of the factors which may affect the mutation of the gene studied. Among the gene differences occurring in the particular cross studied, it can detect only those in which the allele present in the tester parent is dominant to that present in the Cornell stock, only those of relatively large effect upon mutation frequency, and only those rather closely linked to the marker genes used.

EXPERIMENTAL RESULTS

The backcross progeny was tested for seed-color mutation rate in three cultures, 59–101, 102, and 103, each grown from a single ear of $F_1 \times$ Cornell stock. These cultures comprised a total of 224 plants, bearing 41,600 seeds. Among the $R^r R^r$ plants of these cultures, each seed tests an R^r: Cornell gamete; among the $R^r R^g$ plants approximately half of the seeds are tests of R^r: Cornell gametes and half are tests of R^g gametes. The number of colorless seeds found was 32. Each of these, except two that failed to germinate, was confirmed as a mutation by subsequent test. All 30 mutants identified proved to be mutations to r^r, and were therefore presumably the result of mutation in R^r: Cornell gametes.

The estimated number of R^r: Cornell gametes tested in the backcross progenies (number of seeds of $R^r R^r$ plants plus half the number of seeds of $R^r R^g$ plants) was 32,457. The mutation rate for R^r: Cornell gametes in the backcross plants was thus 9.2×10^{-4} (or at most, including the untested mutants, 9.9×10^{-4}), approximately one-half the rate found in the Cornell stock. The 30 mutants observed were produced by 22 plants, the observed mutation frequency in plants that gave more than a single mutant being 3/130, 3/682, 2/76, 2/240, 2/413, and 2/468.

The distribution of the mutants with relation to the segregating marker-genes is shown in Table 1. The same population of 32,457 R^r: Cornell gametes is here classified for each of the 8 marker-genes, except in cases in which the genotype was indeterminate. In such cases the plants concerned are omitted. The only case of serious difficulty in classification was for *j* among plants with *B*. The expression of *j* (japonica striping) in these cultures was restricted almost wholly to the leaf sheaths, and the development of anthocyanin in the leaf sheaths in plants of the *B b* class made classification for *j* doubtful. The *j* classification was therefore made only in the *b b* class.

TABLE 1

MUTATION FREQUENCY OF R^r: CORNELL IN RELATION TO LINKAGE-TESTER GENES

FIRST BACKCROSS

b Su Pr y-pl j Wx R^r (Cornell stock)/*B su pr Y-Pl J wx* R^g × Cornell stock

Genotype	59 101		59 102		59–103		Total		Mutation rate	L. E.
	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	(× 10^{-4})	
B b	30	4/4,270	37	4/5,504	32	4/3,887	99	12/13,661	8.8	15.3 - 4.5
b b	37	6/4,900	41	8/6,396	42	4/7,059	123	18/18,355	9.8	15.5 5.9
Su Su	42	9/6,279	40	4/6,159	40	4/6,295	122	17/18,733	9.1	14.5 5.3
Su su	25	1/2,891	42	8/5,890	35	4/4,943	102	13/13,724	9.5	16.2 - 5.0
Pr Pr	34	3/4,232	45	7/6,516	28	3/4,291	107	13/15,039	8.6	14.8 4.6
Pr pr	38	7/4,938	37	5/5,533	47	5/6,947	117	17/17,418	9.8	15.6 5.7
Y y	33	6/5,138	42	9/6,361	37	7/6,145	112	22/17,644	12.5	18.9 7.8
y y	34	4/4,032	40	3/5,688	38	1/5,093	112	8/14,813	5.4	10.6 2.3
Pl pl	27	7/4,424	42	8/6,845	40	4/5.447	109	19/16,716	11.4	17.8–6.8
pl pl	40	3/4,746	40	4/5,204	34	4/5,775	114	11/15,725	7.0	12.5 3.5
J j	17	6/2,490	25	7/4,485	25	3/4,329	67	16/11,304	14.2	23.0 8.1
j j	20	0/2,410	16	0/1,643	16	1/2,698	52	1/ 6,751	1.5	8.3 -0.0
Wx Wx	32	5/4,362	39	7/5,742	32	6/4,807	103	18/14,911	12.1	19.0 - 7.2
Wx wx	35	5/4,808	43	5/6,307	43	2/6,431	121	12/17,546	6.8	12.0–3.5
R^r R^r	36	10/6,133	47	9/8,802	46	8/8,378	129	27/23,313	11.6	16.9–7.6
R^r R^g	31	0/3,037	35	3/3,247	29	0/2,860	95	3/ 9,144	3.3	9.6–0.7

The results show suggestive differences in mutation rate associated with the marker genes *J*, R^g and *Y*, and possible differences of smaller extent asociated with *Pl* and *wx*.

The statistical significance of these differences may be estimated by the use of Stevens' table of the limits of the expectation, for the binomial and Poisson distributions (Stevens, 1945) as described in the preceding paper. The customary method of estimating sampling fluctuations by the use of the standard error may be very inaccurate in the case of mutation rates, as Stevens (1942) has shown, since the assumption of normality of the distributions is not warranted. This error may be avoided by using the actual ranges expected, as determined from the binomial distribution. The limits of the expectation for various values of P are given in the table cited.

The method of estimating the significance of differences in mutation rate in the contrasted classes may be illustrated as follows, in the case of the comparison of *Y y* vs. *y y* plants: If the difference in genotype were without effect upon mutation of *R'*, the expected number of mutants in the *Y y* class would be 16.3 ($30 \times 17,644/32,457$); in the *y y* class 13.7. The numbers of mutants observed in the two classes were 22 and 8, respectively. We may determine directly from the table whether the smaller class (the *y y* class in this case) has significantly less than its proportionate share of the total of 30 mutants. According to the table, for $n = 8$ and for $p = 8/30 = .267$, the limits of the expectation at the $P = .05$ level of significance are 3.7–13.8. The expected number of mutations, 13.7, is thus barely within the limits of sampling fluctuation at this level of probability, and the difference therefore is not quite significant by conventional standards.

The differences associated with *Pl* and *wx* are much less significant, the former being within the limits of sampling fluctuation at the $P = .2$ level, and the latter only slightly beyond these limits.

The difference associated with *j* is highly significant, as judged by this standard. The observed mutation rate in *J j* plants was 14.2, and in *j j* plants was 1.5. The expected number of mutants in the *j j* class is beyond the limit of the expectation not only at the $P = .05$ level but at the $P = .01$ level. The indication of a nearly 10-fold increase in mutation rate associated with *J* is of course very approximate, since the mutation rate in $j\,j$ plants is based upon a single mutation, and is thus subject to a very high sampling error.

The difference associated with R^g is also significant. The mutation frequency observed in $R^r R^g$ plants was less than one-third as high as that in $R^r R^r$ plants. The expected number of mutants in the $R^r R^g$ class is beyond the limit of expectation at the $P = .05$ level, but not at the $P = .01$ level.

There may still be some material underestimate of sampling error in the determinations of significance given above. These determinations would be strictly accurate for samples from a homogeneous population. Obviously a population in which genetic comparisons are made can not be homogeneous, and comparisons to determine the effect of a single factor or complex can be made only by including in each class a large enough number of plants to reduce the effect of other segregating factors to a negligible quantity. The backcross progeny with which we are here concerned is apparently segregating for large differences in mutation rate linked with J, R^g, and possibly Y, and presumably also for similar differences determined by other chromosome segments not genetically marked. If these effects are cumulative, there must be very large differences in potential mutation rate between the individual plants. The extent to which plant variability reduces the significance of the differences is not measured in the estimates given above. To illustrate this error, suppose that in the data for $Y\,y$ vs. $y\,y$ plants, the 17,644 gametes tested to represent the $Y\,y$ group came from 4 plants of the backcross progeny, while the 14,813 gametes representing the $y\,y$ group came from 4 others. This would be technically possible if male gametes rather than female gametes were under test. Now obviously a major factor determining the result of the comparison would be the chance selection of the four plants in each group. The significance of a difference between the two groups could not be estimated without considering the variations in mutation rate in the individual plants. When the test is made on female gametes, and thus limited to a small and variable number of gametes per plant, the mutation rates of individual plants are determined from quite insignificant samples, and therefore a significant estimate of the sampling error arising from individual plant variability can not be made. It may be supposed that the sampling error from plant variability is negligible when the rates in the two classes are based, as in this case, on the results from more than 100 plants each. But this is only a guess, since the extent of the plant variability is unknown. It should be noted also that, in the case of the difference associated with *J*, the number of plants tested was only about half as large as in the other comparisons.

Since later trials confirmed the significance of the *Y* effect, the relation of mutation frequency to the various combinations of *Y* and *Pl* is of interest. This is shown in Table 2.

TABLE 2

MUTATION FREQUENCY OF *Rr*·CORNELL IN RELATION TO *Y* AND *Pl*

FIRST BACKCROSS

	59-101		59-102		59-103		Total		Mutation rate	L. E.
	No. plants	Mutation frequency	No plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	($\times 10^{-4}$)	
Y Pl/y pl	20	6/3,410	30	7/4,660	27	4/4,247	77	17/12,317	13.8	22.1–8.0
Y pl/y pl	13	0/1,728	12	2/1,701	10	3/1,898	17	5/ 5,327	9.4	22.0–3.0
y Pl/y pl	7	1/1,014	12	1/2,185	13	0/1,200	32	2/ 4,399	4.5	16.4–0.6
y pl/y pl	27	3/3,018	28	2/3,503	25	1/3,893	80	6/10,414	5.8	12.5–2.1

If it may be assumed that the effect is due to a single modifier gene, the data suggest that this gene is closer to *Y* than to *Pl*. The higher mutation rate associated with *Pl* is due entirely to the plants which received from the F_1 parent a *Y*-*Pl* chromosome; those which received a *y Pl* crossover chromosome show no increase in mutation rate over those which received the *y pl* chromosome.

There is some slight indication that the gene-sequence is Y-M_v-Pl (using the symbol M_v to designate the postulated mutation modifier). The reduced mutation rate in plants which receive the *Y pl* crossover chromosome, in contrast to those with *Y Pl*, is in agreement with this supposition. But since the reduction is quite insignificant, the gene-sequence M_v-*Y*-*Pl* is almost equally likely.

The fact that the 7 chromosome segments tested included three with pronounced effects upon mutation frequency must mean that there is a rather large number of genes with such effects, for these 7 segments represent a rather small sample of the total genotype. The fact that the number of modifiers is large does not mean that the effect of the individual modifier must be small, since the effects may occur in either direction. Actually the results indicate that the effects of the single modifier may be surprisingly large.

It is noteworthy that of the three segments from the linkage tester stock that appear to affect the mutation rate of R^r: Cornell, two are associated with increased mutation frequency. These two mutation-favoring segments were present in every plant of the F_1, which nevertheless showed sharply reduced mutation rate of R^r: Cornell. Presumably, in the F_1 plants, their effect was over-ridden by other segments from the tester stock with mutation-reducing effect. Of these, only one happened to be included among those identified by linkage with a marker gene.

The possibility of extracting strains of very high mutation rate depends upon the extent to which these modifier effects are cumulative. When two mutation-favoring segments are brought together, their effect in combination will not necessarily be an augmented increase in the mutation rate. Possibly the modifiers may accomplish their effects in the same way, and the plant with either one may yield mutations at as high a rate as the plant with both. The data available are not extensive enough to show the mode of interaction, but some indication may be secured from this backcross, segregating for the three mutation-favoring segments marked by *Y*, *J*, and R^r. Since only about half of the population could be classified for *J*, the data are further reduced in comparisons involving this effect. The data for the various combinations are given in Table 3.

TABLE 8

INTERACTION OF HIGH-MUTATION SEGMENTS R^r, Y, AND J

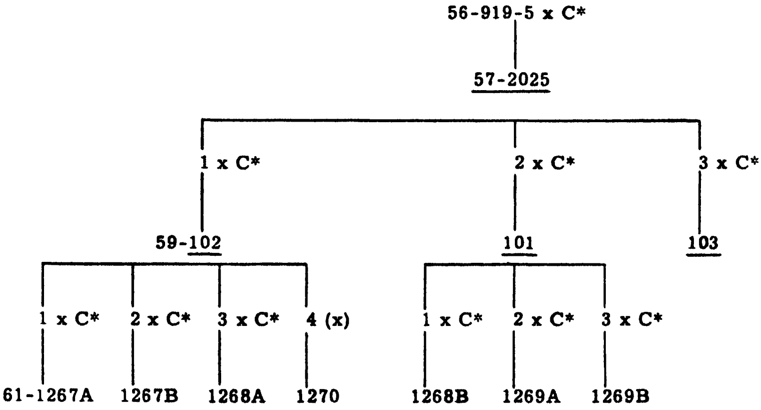
FIRST BACKCROSS

	No. plants	Mutation frequency	Mutation rate	L. E.
			(× 10^{-4})	
R^r Y	66	21/12,819	16.4	25.1–10.1
R^r y	63	6/10,494	5.7	12.5– 2.1
R^g Y	46	1/ 4,825	2.1	11.5– 0.1
R^g y	49	2/ 4,319	4.6	16.7– 0.6
Total	224	30/32,457	9.2	132.1– 6.2
R^r J	41	15/ 8,609	17.4	28.7– 9.8
R^r j	21	1/ 3,384	3.0	16.5– 0.1
R^g J	26	1/ 2,695	3.7	20.7– 0.1
R^g j	31	0/ 3,367	0	10.9– 0
Total	119	17/18,055	9.4	150.8– 5.5
Y J	37	12/ 7,232	16.6	29.0– 8.6
Y j	26	1/ 3,825	2.6	14.6– 6.6
y J	30	4/ 4,072	9.8	25.2– 2.7
y j	26	0/ 2,926	0	12.6– 0
Total	119	17/18,055	9.4	150.8– 5.5

These data indicate that the effects of these three segments are cumulative. For example, among the $R^r R^r$ plants the presence of Y is distinctly favorable to mutation, and among $Y\,y$ plants the presence of R^r is distinctly favorable. Similarly, the addition of the mutation favoring segments in other combinations is in general favorable to mutation, so far as the scanty data permit the test to be made. Apparently, on the average, a plant of high mutation rate is increased in mutation frequency by the addition of a given segment in as great proportion as in a plant of low mutation rate.

The chromosome segment marked by the gene *a* could not be tested for relation to R^r mutation frequency, since the plants of the backcross progeny could not be classified for *A* constitution. The only indication of the relation of this segment to the mutation rate is the *A* constitution of the plants which yielded mutations, which was determined by a special progeny test of these plants. Presumably about half of the plants of the backcross progeny were *A A* and about half *A a*. If the *a* segment has a pronounced effect upon mutation frequency, the mutations should occur predominantly in plants of one or the other of these two classes. The test was made for 21 of the 22 plants that yielded mutations, the other plant being untestable because the residue of viable seed was inadequate. Of the 21 plants, 7 were *A A* and 14 *A a*. The *A A* plants yielded a total of 12 mutations, the *A a* plants 17 mutations. The ratio of 12:17 in the contrasted classes would not be a significant deviation from equality, if the number of gametes tested in the two classes were equal. The excess of *a a* plants among the mutating individuals is suggestive of a possible relation, and it is interesting to note that the linkage suggested would be another case of an effect favorable to mutation in a chromosome segment from the tester parent.

Some further evidence on the relation of the segments marked by *Y-Pl*, *J*, and R^g was secured from progenies of the next generation. These included progenies produced from six plants of the first backcross which were backcrossed again to the Cornell stock, and from one which was self-pollinated. The relationship of the cultures studied is shown in Figure 1.



*Cornell stock .

FIG. 1. Relationship of cultures tested for mutation.

The seven plants of the first backcross that were used as parents of cultures 1267A–1270 were wholly unselected as to mutation rate and genotype. Mutation frequency was determined in all these progenies. The mutation frequency in the second backcross cultures is shown in Table 4.

TABLE 4

MUTATION FREQUENCY OF R^r:CORNELL IN RELATION TO LINKAGE-TESTER GENES

SECOND BACKCROSS CULTURES

Culture	Genotype of Parent			Mutation frequency	Mutation rate	L. E.
	Y-Pl	J	R			
					(× 10-4)	
61–1267A	y-Pl/y-pl	j j	$R^r R^g$	7/8,538	8.2	16.9–3.3
1267B	Y-Pl/y-pl	j j	$R^r R^r$	2/3,388	5.9	21.3–0.7
1268A	y-pl/y-pl	j j	$R^r R^g$	0/2,820	0	13.1–0
1268B	Y-Pl/y-pl	j j	$R^r R^r$	2/3,147	6.4	22.9–0.8
1269A	y-pl/y-pl	j j	$R^r R^g$	2/2,826	7.1	25.6–0.9
1269B	y-Pl/y-pl	J j	$R^r R^r$	3/5,210	5.8	16.8–1.2

All the second backcross progenies were rather low in mutation rate, and none yielded a large enough number of mutations to provide significant confirmatory tests. Culture 1267A, which yielded 7 mutations, was the progeny of a plant of genotype $R^r R^g$, and gives some slight confirmatory evidence for the R relation. Of the 7 mutations, 6 occurred in R^rR^r plants and only 1 in an $R^r R^g$ plant. In the other culture of this genotype which yielded mutants, 1269A, both of the mutants occurred in $R^r R^r$ plants. The totalled mutation frequencies for R^r gametes in these two cultures were 8/6511 in $R^r R^r$ plants and 1/4853 in $R^r R^g$ plants. This is in agreement with the results on the R relation in the first backcross, and although the numbers are too small to be significant in themselves, they have considerable weight as confirmatory evidence.

No other data bearing on the R relation in the cross are at hand, since the self-progeny still to be discussed was produced by an $R^r R^r$ plant. There are, however, comparable data from a cross of the Cornell stock with another R^g tester. The F_1 of this cross (reported in Table 3 of the preceding paper) yielded r mutations with a frequency of 9/79,858, including 6 r^r, 2 r^g, and 1 undetermined. The indicated mutation rate for R^r:Cornell gametes was therefore 1.7 (6.75/39,929), a pronounced reduction from the rate characteristic of the Cornell stock. Backcross progenies produced by crossing three F_1 plants with Cornell stock, yielded 22 r^r mutations and 1 r^g mutation. The frequency of r^r mutations among R^r gametes tested was 20/15,036 in the $R^r R^r$ plants and 2/6,725 in the $R^r R^g$ plants. The difference in mutation rate is significant at the $P = .05$ level. The result is in agreement with that secured from the cross with the multiple linkage tester stock, in showing a substantial decrease in the seed-color mutation rate of R^r:Cornell in plants heterozygous for this chromosome segment, as compared to their homozygous sibs.

The plant which was selfed to produce culture 61–1270 was an $R^r R^r$ plant heterozygous for *Y-Pl*/*y-pl* and *J j*. This culture included 96 plants, bearing 34,344 seeds, and yielded 52 mutations (rate 15.1). The plants were classified phenotypically for *Pl* and *J*. The mutation frequencies determined for plants of these phenotypic classes are shown in Table 5.

TABLE 5

MUTATION FREQUENCY OF R^r:CORNELL IN RELATION TO LINKAGE-TESTER GENES

Culture 61-1270 (Self of an R^r R^r *Y-Pl y-pl J j* Plant of First Backcross)

Phenotype	No. plants	Mutation frequency	Mutation rate	L. E.
			(× 10^{-4})	
Pl	68	44/26,285	16.7	22.5-12.2
pl	28	8/ 8,059	9.9	19.5- 4.3
J	77	42/26,099	16.1	21.8-11.6
j	19	10/ 8,245	12.1	22.3- 5.8

The data from a self-progeny are not directly comparable with those from a backcross progeny. In a backcross progeny the comparative mutation rate in *Y y* and *y y* plants, for example, measures the effect of the genes of the *Y* segment plus those of the *y* segment, as compared to two doses of the genes of the *y* segment. If the genes modifying mutation rate are strongly dominant, the *Y* segment may show a significant effect in this comparison, even though the opposite segment carries modifiers of equal effect on mutation rate. For example, suppose that the *Y* segment carries a dominant modifier *M* which doubles the mutation rate, while the *y* segment carries at another locus a dominant modifier *N* of similar effect. In the backcross progeny, the comparison of *Y M n/y m N* versus *y m N/y m N* plants (neglecting crossovers) would show the increased mutation rate due to *M*, superimposed upon the rate resulting from the action of *N*, which would be effective in both classes. But in the self-progeny, about ⅓ of the *Y* plants would be *Y M n/Y M n*, and would show a depressed rate due to the absence of *N*. Similarly, with modifiers of weak dominance, the results in backcross and self-progenies would not be comparable, due to the different proportions of plants with single and double doses of the genes concerned. Another differential factor concerns the reduction in detectable effect on mutation, due to crossing over between the marker gene and the modifier gene. This would be expected to be greater in the self-progeny than in the backcross progeny.

The data of Table 5 show a material difference in mutation rate associated with *Pl*, which is in the expected direction. It is not significant at the $P = .05$ level, but taken in connection with the results for the preceding generation it strongly indicates the validity of the linkage. The *Pl* relation is further discussed below.

The difference in mutation rate associated with *J*, as indicated in Table 5, is wholly insignificant. With equal mutation rate in *J* and *j* plants, the number of mutants expected in the *j* class would be only 12.5, and the total of 10 mutants observed is in reasonably close agreement. Thus the indication of a strong mutation-favoring effect of the *J* segment, shown by the first backcross generation, is not confirmed in this self-progeny.

Does this contradict the earlier indication of a *J* relation? Aside from the possibility that the *J j* plant which was the parent of culture 1270 may have been a crossover, there is reason to withhold a decision on this point. Among the 19 *j* plants of culture 1270 which in total showed a mutation frequency of 10/8,245, there was included one plant with a mutation frequency of 5/660. If this plant were considered a crossover, and were arbitrarily shifted to the *J* class, the mutation rates for the *J* and *j* classes would be 17.6 and 6.6 respectively, and the difference would be significant at the

$P = .05$ level. There is of course no basis for making this arbitrary reclassification. But the fact that it would have the effect stated emphasizes the importance of plant variability in these comparisons, and shows that the indication of equal mutation rate in the two classes is quite insecure. Even though the high mutation rate of the plant in question were the result, not of crossing over of *j*, but of the over-riding effect of some unusual combination of mutation modifiers, this reservation would still apply. In view of the high sampling error from plant variability, the original indication of the *J* relation in the first backcross also must be considered tentative.

It would be desirable, if possible, to exclude from the comparison the homozygous dominant plants, both for *Pl* and for *J*, in order to avoid in part the complications discussed above. This is not feasible for *J*, which is identified by a mature plant character, but it may be accomplished with *Pl*, by using as a basis of classification the seedling root-color effect. When the seeds are germinated in darkness, the roots of *Pl* (R^r) plants develop anthocyanin while the roots of *pl* (R^r) plants do not. This classification is often unsatisfactory because of poor root coloration, but in this material it gave clear separations.

Since all the plants of the culture were pollinated by a *y y pl pl* pollen stock, it was possible to classify the plants as *Y Y*, *Y y*, and *y y* by seed color and as *Pl Pl*, *Pl pl*, and *pl pl* by root color. This classification was made for all plants which yielded 200 seeds or more. Of the 96 plants, 70 produced 200 seeds or more, and the total was 31,031 seeds, including 46 mutants (mutation rate 14.9). The distribution of the mutants with relation to homozygous and heterozygous *Y* and *Pl* is shown in Table 6.

The result clearly shows that the increase in mutation rate associated with both *Pl* and *Y* is reduced in apparent effect in the self-progeny by the inclusion of the homozygotes *Pl Pl* and *Y Y*. The comparative mutation rates for *Pl* and *pl* plants in the 70 plants are 16.1 and 10.3 respectively, but when the 18 *Pl Pl* plants are excluded, the rates

TABLE 6

MUTATION FREQUENCY OF R^r:CORNELL IN RELATION TO HOMOZYGOSITY OF Y-Pl SEGMENT
Culture 61-1270 (Self of an *R^r R^r Y-Pl y pl J j* Plant of First Backcross)

	Pl Pl		*Pl pl*		*pl pl*		Total			
	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	Mutation rate	L. E.
									(× 10-4)	
Y Y	8	1/3,625	5	4/ 2,260	2	0/ 430	15	5/ 6,315	7.9	18.5– 2.6
Y y	6	6/2,505	18	22/ 8,350	4	3/2,090	28	31/12,945	24.0	34.1–16.2
y y	4	1/1,840	14	5/ 5,665	9	4/4,266	27	10/11,771	8.5	15.6– 4.1
Total	18	8/7,970	37	31/16,275	15	7/6,786	70	46/31,031	14.8	19.8–10.8
Mutation rate (× 10-4)		10.0		19.0		10.3		14.8		
L. E.		19.8–4.8		27.1–12.9		21.2–4.1		19.8- 10.8		

compared are 19.0 and 10.3. The corresponding change for the Y relation is from 18.7 : 8.5 to 24.0 : 8.5.

Considering only the data from plants not *Y Y* or *Pl Pl* (that is, the cells of columns *Pl pl* and *pl pl*, rows *Y y* and *y y* of Table 6) the results are roughly comparable to those of the first backcross, as shown in Table 1. The data are as follows:

	Mutation frequency	Mutation rate
Y y	25/10,440	23.9
y y	9/ 9,931	9.1
Pl pl	27/14,015	19.3
pl pl	7/ 6,356	11.0

The difference between the *Y y* and *y y* classes is significant at the $P = 0.05$ level: that between the *Pl pl* and *pl pl* classes is not. The agreement between this and the preceding progeny strongly indicates the significance of the linkage for both markers.

As in the preceding generation, the linkage is closer with *Y* than with *Pl*. The relation of mutation frequency to *Y* and *Pl* combinations, comparable to the first backcross data of Table 2, is as shown below.

As before, *Pl* fails to show any favorable effect upon mutation in the *y Pl* crossovers, indicating that the mutation-modifying gene or genes are closer to *Y* than to *Pl*. There is again a slight indication favoring the order *Y-M-Pl*

	Mutation frequency	Mutation rate
Y Pl/y pl	22/8,850	26.3
Y pl/y pl	8/2,090	14.4
y Pl/y pl	5/5,665	8.8
y pl/y pl	4/4,266	9.4

over *M-Y-Pl,* since crossing over between *Y* and *Pl* reduces the mutation-favoring effect of *Y.*

The reduced mutation rate in plants homozygous for *Y Y* or *Pl Pl* suggests the possibility of a dominant mutation-favoring modifier in the *y-pl* segment from the Cornell stock. Assuming a single dominant gene to be responsible for the increase in mutation rate of *Y Pl/y pl* plants over *Y Pl/Y Pl* plants, the data of Table 6 may be used similarly to indicate its effect and locus. Disregarding crossovers, this modifier (here designated *N*) is present as *N N* in the class *y pl/y pl, N n* in the class *Y Pl/y pl,* and *n n* in the class *Y Pl/Y Pl.* Corresponding data for the effect of the heterozygote compared to the homozygous recessive are therefore given by the cells of columns *Pl Pl* and *Pl pl,* rows *Y Y* and *Y y* of Table 6. These show the following relations:

	Mutation frequency	Mutation rate
Y y	28/10,855	25.8
Y Y	5/ 5,885	8.5
Pl pl	26/10,610	24.5
Pl Pl	7/ 6,130	11.4

The difference in mutation rate between *Y y* and *Y Y* plants is significant at the 0.05 level, and the difference between *Pl pl* and *Pl Pl* is just short of significance. This linkage also is closer with *y* than with *pl.*

The relation to *y* and *pl* recombinations is as follows:

	Mutation frequency	Mutation rate
y pl/Y Pl	22/8.350	26.3
y Pl/Y Pl	6/2,505	24.0
Y pl/Y Pl	4/2.260	17.7
Y Pl/Y Pl	1/3,625	2.8

The effect of *y* upon mutation rate is substantially unaffected by crossing over between *y* and *pl,* indicating the order *N-y-pl,* or the order *y-N-pl* with *N* much closer to *y* than to *pl.* The effect of *pl* is possibly reduced in *Y pl* crossovers, as compared to *y pl* non-crossovers, but is not wholly lost, as might be expected if the order is *N-y-pl.*

Obviously these indications of the locus of *M* and *N* with reference to *Y* and *Pl* are very questionable, because of the small numbers involved in the comparisons. It is probable that both modifiers are closer to the *Y* locus than to the *Pl* locus, and possible that both are located between *Y* and *Pl.* The considerations suggesting their loci are based on the assumption that the effects of the chromosome segments indicated are the result of single factors in each case, and that these factors are dominant in effect to such a degree that the effect of *M m,* for example, is substantially equal to the effect of *M M.* The possibility that *M* and *N* are allelic is not excluded by the evidence, but in this case the effect on mutation rate must be substantially greater for the heterozygote *M N* than for either homozygote, *M M* or *N N.*

DISCUSSION

The results indicate that the frequency of the gene mutation studied, seed color mutation of R^r: Cornell, is susceptible to a surprisingly high degree to the action of modifying genes. The marker-genes of the linkage tester stock used covered effectively only a relatively small part of the total genotype; the populations tested were large enough to identify only modifiers of rather strong effect; and the experimental design was such as to detect only those modifiers in which the allele derived from the tester stock was dominant to the allele derived from the Cornell stock. Yet three of the seven marked segments proved to have detectable effects on mutation frequency. Presumably various other modifiers of similar action were segregating in the backcross tested, but were indistinguishable because they were unlinked, or were not closely enough linked, with the marker genes used. The presence of additional low mutation modifiers is indicated by the extreme reduction in mutation frequency in the F_1, despite the presence in all the plants of the high-mutation modifiers demonstrated in the backcross generation. Presumably also there is a corresponding class of modifiers in which the allele present in the Cornell stock is dominant to that present in the tester stock. These would be identifiable in the converse backcross, $F_1 \times$ tester stock, though it is unlikely that the mutation rate in this cross would be high enough to make their identification feasible.

These alleles are referred to as dominants on the basis of the comparison of the heterozygote with only one of the two homozygotes. They would not necessarily appear as dominants if the effect of the other homozygote were known. For example, the gene *Dt,* which has a very pronounced effect on the frequency of the mutation a → A, shows disproportionately increasing effects with increasing gene dosage. The number of mutations produced in endosperm tissue by the *Dt Dt Dt, Dt Dt dt,* and *Dt dt dt* genotypes, respectively, are in the approximate ratio 17:3:1 (Rhoades, 1941). Now if a gene of comparable action on R → r mutation, here designated *D,* were present in the Cornell stock, and its allele *d,* comparable to *dt,* were present in the tester stock, the backcross ($F_1 \times$ Cornell stock) would include plants of genotype *D D* and *D d.* The mutation rate would be lowered in the *D d* class, and the gene *d* would appear as a dominant modifier reducing mutation frequency.

In the case of the R^g segment, the possibility must be considered that the reduction in mutation frequency may be brought about by some direct interaction of the R^g and the R^r gene rather than by a modifier linked to R^g. The possibility that mutation of *R* may be associated with crossing over at or near this locus is not excluded, although it has been shown (Stadler, 1948) that the mutation is not associated with crossing over between *R* and *G.* Further data on the relation of *R* mutation to crossing over will be submitted shortly. Proof that the effect is due to a modifier rather than to R^g requires the demonstration of a crossover separating the two genes.

The extent of the increase or decrease in mutation frequency due to the action of the individual modifiers can be estimated only roughly. The ratios of mutation frequency in the contrasted classes in the backcross progeny were 1.0 : 2.3 for the *Y* segment, 3.5 : 1.0 for the R^g segment, and 1.0 : 9.5 for the *J* segment. But these ratios are based upon a total of only 30 mutants and are subject to high sampling errors. The evidence on the extent of the *J* effect is especially weak, since it is based upon only 17 mutants, 16 of which occurred in *J j* plants and only 1 in a *j j* plant. The observed yield of 1 mutant in the *j j* class does not significantly contradict a frequency of nearly 5 mutants in this class. Although the difference in mutation rate between the *J j* and *j j* classes is clearly significant (assuming the classes to be comparable) the result does not contradict the possibility of an increase in mutation rate as low as 3-fold for the *J* segment. The 2.3-fold increase found for the *Y* segment is in good agreement with the increase indicated for this segment in the self-progeny (Table 6), and the reduction in mutation rate associated with the R^g segment in the backcross progeny (3.5 : 1.0) is fully matched by the reduction associated with the R^g segment from the McBaine stock (4.4 : 1.0). In view of the indication that modifying factors may be very numerous, the differences in mutation rate associated with specific segments are not necessarily to be ascribed to the effects of single modifier genes. Detailed studies of the individual segments will be necessary to determine whether separable genes are involved and to indicate the loci of the modifiers. The 3- to 4-fold modification of the mutation rates indicated for the modifiers would be substantially increased by evidence of frequent crossing over between modifier and marker.

The indication that gene mutation is so readily affected by diverse modifiers encourages the hope that the mutation process may be subject to experimental control, for each modifier must produce its effect by altering in some way conditions which affect the probability of mutation. It is plausible to suppose that the occurrence of gene mutation is determined by chemical conditions within the cell. But even if the necessary conditions were precisely known, it would probably be impossible in most cases to approximate them by chemical treatments, because of the insulating mechanisms of the living organism. Chemical alterations effected by gene action escape this limitation.

If the indication of large numbers of modifiers with pronounced effects on mutation frequency is substantiated by further investigation, these modifiers may be very useful in the study of the nature of gene mutation. Two approaches may be mentioned:

(1) The extraction of strains in which specific gene mutations are made frequent enough to permit direct experimental study of the phenomenon.

A major obstacle to the experimental study of the mutation process has always been the very low frequency of mutation. The study of X-ray effects on mutation was originally motivated chiefly by the hope that gene mutation might thus be made frequent enough for effective experimental investigation. This hope has not been realized. The frequency of point mutations was greatly increased by the treatment, but critical study of the mutations induced has shown that various extra-genic alterations may closely simulate gene mutation. There are no general criteria by which gene mutations may be distinguished from other Mendelizing variations among a group of induced point mutations at miscellaneous loci. Consequently, the high general mutation rate secured by the use of X-ray treatment does not provide suitable experimental material for the study of the reaction in which a gene is converted to an alternative form. Further, the recognition of the variety of extra-genic phenomena simulating gene mutation makes it seem doubtful that the study of this reaction could be made effectively by means of any experiments on the general mutation rate, for spontaneous mutations also may include extra-genic alterations. The investigation of gene mutation seems to require the intensive study of the mutation of specific genes especially suitable for critical analysis.

This of course intensifies the difficulty arising from the rarity of the mutations.

The results here reported suggest that it may be possible to bring the frequency of occurrence of the specific mutation here studied to a surprisingly high level, by combining favorable modifier genes. The Cornell stock itself represents an unusual combination of modifiers favorable to the $R^r \rightarrow r^r$ mutation, and the results indicate that its high mutation rate is due to this fact rather than to any inherent instability of the R^r: Cornell gene. The further increase in mutation frequency resulting from the addition of the *Y*-linked and *J*-linked modifiers, if maintained proportionately at high mutation levels, and if cumulative in effect, might make possible a considerable multiplication of the characteristic Cornell rate. This might be accomplished by repeated backcrossing to the Cornell stock, with retention of the *Y* and *J* segments. In some cases, as appears to be probable for the *Y* segment, the substitution of a segment carrying a mutation-favoring modifier might fail to increase mutation rate because the Cornell stock may carry an equally favorable modifier in the corresponding segment.

It should be possible to make use of unmarked segments favorable to mutation, as well as the marked segments, by selection based upon direct mutation rate determinations in the individual segregating plants. Using mutation frequency in male rather than in female gametes, it is feasible to make significant mutation rate determinations for individual plants. If mutation modifiers are as frequent as these results suggest, continued backcrossing with selection based on individual tests should quickly isolate strains of greatly increased mutation rate.

(2) The use of the various mutation modifiers as differential reagents, so to speak, in the analysis of gene differences.

The known alleles of *R* include several groups of distinctive action, and some of these are known to mutate in distinctive ways. In the r^{ch} group, in which much increased pigmentation of the pericarp and certain other tissues is superimposed upon the usual plant-color pattern, the two alleles studied yield plant-color mutations in which some of the pericarp-color and plant-color effect is retained. On the contrary, in the various alleles of the Cornell group, plant-color mutations have in all cases resulted in complete loss of plant-color, including the slight pericarp-color effect which occurs in this group. In the Cornell group, plant-color mutations occur independently of seed-color mutations. But in certain alleles of the Winnebago group, which is characterized by dilute plant-color effect, there is limited evidence suggesting that complete loss of seed-color and plant-color effect occurs simultaneously in a single mutational step.

Previous studies, as noted in the preceding paper, have indicated that the modifier complexes affecting the mutation rates of different R^r alleles are different, and that the modifier complex affecting the seed-color mutation rate of R^r: Cornell is different from that affecting the plant-color mutation rate of the same allele. The occurrence of a mutation for plant-color in R^r: Cornell greatly reduces the subsequent frequency of mutation for seed color (Stadler, 1946 and unpub.), but whether or not this reduction in seed-color mutation rate involves a change in its relations to mutation modifiers is unknown.

A priori it may be supposed that a group of mutation modifiers identified by their effects upon the frequency of a given mutation may include modifiers of various degrees of specificity, ranging perhaps from modifiers which may affect mutation frequency in general to modifiers which may affect the frequency of only a single type of mutation of a single allele. The seed-color mutation of R^r: Cornell may thus serve as a convenient detector for the identification of a collection of mutation modifiers, among which there may be many which affect various other mutations at the *R* locus and perhaps at other loci. The testing of an adequate sample of such modifiers upon the various alleles of *R* in their various types of mutation might yield evidence of systematic relations in the response of these alleles to the conditions affected by the various modifiers. This would provide a basis for analytical investigation of the mutation process.

SUMMARY

1. The frequency of seed-color mutation of R^r: Cornell, which occurs at the rate of 18.2×10^{-4} in the Cornell stock, was reduced to 0 in the F_1 of a cross with a multiple linkage tester stock. The number of R^r: Cornell gametes tested for mutation in the F_1 was sufficient to have yielded 22 mutations at the rate characteristic of the Cornell stock.

2. The backcross, F_1 × Cornell stock, yielded 30 mutations (rate 9.2×10^{-4}). The incidence of these mutations in relation to the segregation for the various marker genes indicates the presence of dominant genes favoring seed-color mutation of R^r: Cornell in the *Y*-marked segment and the *J*-marked segment of the tester stock. Similarly, the R^g-marked segment shows an effect unfavorable to the occurrence of the mutation.

3. The increase in mutation rate associated with the presence of the *J* segment appears to be highly significant statistically ($P < .01$). Since the statistical comparison assumes that sampling error due to individual plant variability is negligible, and since the results of the experiment as a whole indicate that plant variability may be very great, the evidence for the occurrence of this modifier is not considered conclusive.

4. The increase in mutation rate associated with the *Y*-segment was not quite significant in the backcross generation, but was clearly confirmed in the self-progeny of a backcross plant. The ratio of mutation rate in the *y y* vs. the *Y y* class was 1.0 : 2.3 in the backcross generation and 1.0 : 3.5 in the following generation.

5. The decrease in mutation rate associated with the R^g segment was significant in the backcross generation ($P < .05$) and was supported, in numbers inadequate for significance, in the second backcross generation. The location of a modifier in this segment is supported also by sig-

nificant evidence of the same sort from a cross of Cornell with another stock. The ratio of mutation rate in the $R^r R^r$ vs. the $R^r R^g$ class was 3.5 : 1.0 in the backcross generation of the cross with the linkage tester stock, and 4.4 : 1.0 in the backcross generation of the other cross mentioned.

6. The effect of the modifiers upon mutation frequency appears to be cumulative, so far as may be judged from the limited data.

LITERATURE CITED

Rhoades, M. M.
1941. *Cold Spring Harbor Symposia,* 9: 138–144.
Stadler, L. J.
1946. *Genetics,* 31: 377–394.
1948. Am. Nat., 82: 289-315.
Stevens, W. L.
1942. *Jour. Genetics,* 43: 301–307.
1945. In Fisher and Yates, Statistical Tables for Biological, Agricultural, and Medical Research, Table VIII.

THE IMPORTANCE OF PLASMOLYSIS AND OSMOTIC PRESSURE IN MAKING NATURAL MOUNTS OF *SPIROGYRA* AND *CLADOPHORA*

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THE word "plasmolysis" is derived from the Greek *plasm* having biological reference to living matter of a cell, and *lysis* signifying a loosening or detachment. Specifically, it refers to the contraction or shrinkage of the protoplasm of a cell due to loss of water by exosmosis. Plasmolysis occurs when a living plant cell is placed in a sugar or salt solution in which the concentration of the solute is greater than that within the cell. The volume of the cell decreases slightly at first, after which the protoplasm (including the chloroplasts) draws away from the cell wall and contracts. If such a cell is then placed in water, the protoplasm gradually regains its original form.

This plasmolytic action can be explained in terms of *osmotic pressure,* which develops as a result of the flow of solvent toward the solution having a higher molecular concentration. The osmotic pressure of the solution is then greater than that of the cell sap. Plant cells usually contain one or more vacuoles. Normally, the osmotic pressure of the solution within the vacuole is such that water tends to enter the cell. When a cell is immersed in a solution having the same osmotic pressure as the cell sap, that solution is said to be isosmotic or *isotonic* with the cell sap. An example of this is the pond water in which fresh-water algae live. A *hypotonic* solution is one whose osmotic pressure is lower than that of the vacuolar sap, whereas a *hypertonic* solution has a pressure higher than that of the sap.

The osmotic pressure increases with the concentration and temperature of the solution. Crystalloids produce a higher osmotic pressure than do colloids. The nature of the separating membrane also determines the extent of osmosis. Since most cells in water absorb the latter, the protoplasm presses against the elastic cell wall and stretches it. When the pressure admitting water into the cell is balanced by the inward pressure of the stretched cell wall, the cell is said to be turgid or in a state of turgor.

When *Spirogyra* or *Cladophora* cells are placed in a solution other than pond water, the osmotic pressure of the solution tends to force water out of the cells. The hydrostatic pressure of the cell sap against the cell wall is known as *turgor pressure.* The inward pressure of the stretched cell wall is equal and opposite to the turgor pressure. When *Spirogyra* is in its natural habitat, its turgor pressure is at a maximum. However, when plasmolysis occurs, the turgor pressure is at a minimum. The osmotic pressure of the surrounding solution is then said to be greater than the osmotic pressure of the cell sap minus the turgor pressure.

Every substance absorbed by a plant cell must pass through two membranes, the cell wall and the plasma membrane. The former is permeable to most dissolved substances. The plasma membrane, or outermost limit of the protoplasm, is semi-permeable in nature, admitting only certain substances. The term is used for convenience, for in most plants no definite structural plasma membrane can be seen under the microscope. In the measurement of osmotic pressure of plant cells collodion membranes, precipitation membranes, parchment paper, and animal bladders have been employed. The latter have osmotic properties similar to that of plant cell walls, while precipitation membranes (usually copper ferrocyanide) give rise to high osmotic properties and are only slightly permeable to many substances. In any event, the membrane acts as a sieve, allowing passage of molecules of the diffusing substances.

A membrane acts as a sieve or " ultrafilter " when it is a porous, heterogeneous substance. The molecules of the solute may then pass only through the pores. If, however, the membrane is homogeneous, the dissolved substance passes through it in solution or is adsorbed at the surface of the membrane. The permeability of the plasma membrane varies considerably during the life of the cell due to changes in external conditions. The higher the temperature, the greater the permeability, up to a certain point. Visible light, ultraviolet rays, radium, and X-rays increase permeability. Hydroxyl groups lower penetrating power, while ethyl and methyl groups increase it.

Some substances enter plant cells very slowly or not at all, while others are readily absorbed. Hydrocarbons and fat solvents pass readily through the plasma membrane, whereas sugars and salts pass more slowly. Weak acids and bases penetrate rapidly, while strong electrolytes penetrate slowly. The more concentrated the external solution, the less water the cell can absorb. The ions of a dissolved substance must also be considered. Sodium and potassium ions increase cell permeability; bivalent cations decrease permeability. Recently sodium, potassium, chlorine, calcium, and other elements have been made radioactive, and their penetration into living protoplasm can be traced by means of the Geiger counter. Atomic research has thus made a significant contribution toward a better understanding of plasmolysis and permeability.

Having thus reviewed the factors involved in plasmolysis and osmosis, their application in attempts to mount *Spirogyra* and *Cladophora* in the natural state will be made more comprehensible.

SPIROGYRA

Spirogyra is an unbranched, filamentous, colonial green alga occurring in large masses in fresh-water ponds and pools. It may be macroscopically distinguished from most other filamentous green algae by its slippery feeling due to the presence of a gelatinous sheath outside the cell wall. Microscopically the cells are cylindrical and attached end to end. The diameter of the cell varies from 25 to 30 microns, while its length is from 200 to 300 microns. The outer wall is about 3 microns thick; in growth it increases in length, but not in diameter.

A thin layer of dense cytoplasm, not over 10 microns thick, lies just inside the wall of each cell, and is held against the wall by the hydrostatic pressure of the cell sap in the central vacuole.

In each cell, characterizing this genus, there is a green, spirally coiled, ribbon-like chloroplast from 5 to 10 microns in thickness. The edges may be smooth, serrated or crenated. The chloroplast does not lie in the center of the vacuole, but is attached to the outer portion of the peripheral cytoplasm, and its coils follow the cylindrical form of the cell. Its specific gravity and refractive index are greater than that of the vacuolar sap. In some species there are two chloroplasts in each cell. Spaced at regular intervals along each chloroplast are small, spherical bodies known as pyrenoids, which function as condensation centers during starch formation. They are connected by a central strand extending along the length of the chloroplast. The chloroplasts of adjacent cells are not connected. They are separated by a common cellular end wall consisting of a central layer of pectose having a cellulose layer on each side of it.

The nucleus, which is often invisible in the living cell, is suspended in the center of the vacuole by thin, dense cytoplasmic strands, each of which extends to the peripheral cytoplasm and terminates immediately beneath a pyrenoid.

The writer considered that it would be useful for college departments of biology to have on hand permanent slides of *Spirogyra* exactly as it appears in the living condition, retaining the shape and natural color of the chloroplasts with no plasmolysis induced. This was easier said than done, for this plant was found to be extremely sensitive to most liquids other than pond water, tap water or distilled water.

The two most important problems, therefore, were (1) to fix the spiral chloroplasts in place, and (2) to retain the chlorophyll and consequently the green color. These factors had to be kept in mind in the selection of a mounting medium. Fixation in alcohol, no matter what the concentration, was found to be useless, since it extracted the chlorophyll and also induced plasmolysis. Therefore alcoholic mounting media, such as euparal, diaphane, and Venetian turpentine, were immediately ruled out, although they were tried just to be sure of their effects.

When plasmolysis occurs in *Spirogyra,* the chloroplast becomes detached or loosened from the peripheral cytoplasm and contracts into a rod-shaped or irregularly rounded mass in the center of the vacuole which loses considerable water. The peripheral cytoplasm itself does not usually withdraw from its proximity to the cell wall; however, with more concentrated solutions it does tend to contract, and a definite space can be seen between the plasma membrane and the cell wall.

Various other fixing agents were tried in different concentrations. Formaldehyde did not extract the chlorophyll, but caused plasmolysis. Osmic and picric acid in various dilutions were found to be useless, for, although they fixed the chloroplasts in place, they removed the natural green color. Dilute acetic acid also caused plasmolysis. At the time that these failures were occurring, other mounting media were considered. In order to mount in Canada balsam, the plant would have to be placed into xylol. To mount in a synthetic resin such as Permount, the *Spirogyra* filaments would have to be placed first into toluene. Unfortunately, both xylol and toluene were found to induce twisting of the filaments as well as marked plasmolysis.

It soon became clear that *Spirogyra* would have to be mounted in a water-soluble medium, since the plant could not tolerate any other medium. Filaments were transferred from pond water to pure glycerine, which is soluble in water. Plasmolysis occurred, but the color was retained. Very dilute glycerine was then used, but plasmolysis occurred immediately. The chemical composition of the chloroplasts was not known, but it was assumed that proteins were present in large percentage. Therefore anything that would fix proteins would probably be the substance or method sought.

It occurred to the writer that heat, if applied suddenly, would tend to fix or coagulate the proteins. A few *Spirogyra* filaments were placed on a slide in a drop of pond water, and the slide was then placed upon a hot aluminum plate. The water evaporated almost immediately, and the chloroplasts remained in spiral form, but they seemed to have lost some color and to have become slightly distorted. After numerous other attempts, success was obtained with hot water. Thus only one method was found to be successful in making permanent slides of *Spirogyra* as it occurs in the natural condition. This method is outlined as follows:

(1) Heat a half-filled beaker of pond water until boiling begins.

(2) Remove from the source of heat and wait until the bubbling subsides.

(3) Drop a small mass of filaments into the water, and allow them to remain for at least 30 minutes.

(4) Transfer to 10 per cent glycerine for 30 minutes, then transfer to 30 per cent, 50 per cent, and 75 per cent glycerine also for 30 minutes each.

(5) Allow the 75 per cent glycerine to evaporate until the liquid is of the consistency of pure glycerine.

(6) Place a drop of the latter on a clean slide. Into it place several small *Spirogyra* filaments, then apply a cover slip. Just enough glycerine should be used as to prevent seepage beyond the edges of the cover slip.

(7) Ring with melted glycerine jelly. (Soak 4 grams gelatin in 30 cc. water, add 30 cc. glycerine, heat, and add a very small crystal of phenol or camphor. Heat to melt before ringing cover slips.)

(8) Ring with gold size (used for affixing gold leaf to windows), and repeat with another coat after the first dries.

(9) Apply a final ring of synthetic resin mounting medium, such as Permount, or use Bell's microscopic cement if available.

Such slides made by the writer one year ago are still in excellent condition. The *Spirogyra* chloroplasts have retained their natural green color and shape, no plasmolysis having occurred. The chief disadvantage of such slides is that the mounting medium, being glycerine, is in liquid form, and may be forced out when excessive pressure is exerted upon the cover slip in using the high power objective carelessly. However, they have served a most useful purpose, and are expected to last for a long period of time.

CLADOPHORA

Cladophora is a branched, filamentous, rough green alga usually found growing attached to objects in shallow fresh water, especially along shores of lakes. Some species, however, are brackish or marine. The diameter of the filaments is greater at the base than at the ends. The cylindrical cells are united end to end. Just inside the thick cell wall is a cylindrical layer of dense, net-like cytoplasm in which are embedded hundreds of small, round or oval chloroplasts. Numerous pyrenoids are usually present. Many nuclei are embedded in the innermost portion of the peripheral cytoplasm beneath the chloroplasts. The large central vacuole is filled with cell sap. In addition there are smaller, regularly spaced peripheral vacuoles, 4 to 24 microns in diameter, in which are stainable, plate-like crystalloids never larger than 8 microns. By macerating filaments in a weak neutral-red solution, these vacuoles and their contained bodies stand out. Zoospore and gamete formation are frequently observed.

The methods described above for *Spirogyra* were tried with *Cladophora*, but plasmolysis occurred more markedly than in the former plant. It was found that *Cladophora* is much more sensitive than *Spirogyra* and plasmolyzed very quickly. When dropped into boiling water, a marked degree of shrinkage occurred, therefore this method of fixation, although used successfully with *Spirogyra,* was considered to be useless in the case of the other plant.

Only one method gave temporary results. Small branches of *Cladophora* were mounted directly from pond water into a 5 per cent gelatin solution on a slide, and a cover slip affixed. For three days the filaments remained natural in appearance, but on the fourth day plasmolysis was found to have occurred. Marked shrinkage had taken place in each cell, so that the green protoplasm in each cell was reduced to a small rectangle in the center of the cell.

It was concluded, after careful study of the results, that plasmolysis had taken place because of the growth of molds and bacterial colonies in the gelatin. However, when substances to prevent mold growth, such as phenol and camphor, were added to the gelatin when mounting the *Cladophora,* the fungicidal and bactericidal substances caused immediate plasmolysis.

Due to the extreme sensitivity of *Cladophora,* it is evident that much additional study and experimentation will be necessary before natural mounts of this green alga can be made without plasmolysis.

LONGEVITY OF THE PARASITIC WASP *HABROBRACON JUGLANDIS* ASHMEAD

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Introductory History

Among animals, insects provide favorable material for a study of longevity and in recent years increasing attention has been focussed on this problem. Rau (1910), investigating the life span of the saturniid moth, *Samia cecropia,* found that the mean length of life of the male was about 11 days, whereas that of the female was only about 8 days. Rau and Rau (1912, 1914) reported that the life span in *Samia cecropia* was not significantly shortened or lengthened by mating, but that low temperatures greatly prolonged their life span.

Lutz (1915) studied the duration of life of *Drosophila melanogaster* in the presence of water, but in the absence of food. The results showed that water increased the mean length of life. Loeb and Northrop (1916), performing the same experiment, stated that the mean duration of life of the flies varied inversely as the temperature between 9 and 34° C. Loeb and Northrop (1917) found that there existed a definite temperature coefficient for the duration of life in *Drosophila* and that the nature of food influenced the duration of life. Northrop (1917) found that the total duration of life in *Drosophila* can be prolonged if the larval period is prolonged by inadequate feeding.

Although *Drosophila* has been a favorite insect for experimental study for many years, the first accurate report on its duration of life was made by Pearl and Parker in 1921. They made a series of reports on the factors influencing the duration of life. They reported that there were wide differences in the life span of different types of *Drosophila* and that the basis of these differences was hereditary rather than environmental. This opened a new period in the history of investigations on longevity, particularly in entomology. Pearl and Parker (1922) found that females lived longer than males at all densities, when full-fed. On the contrary, Pearl and Parker (1924) observed that under starvation the mean duration of life was almost the same in the vestigial as in the wild-type.

Alpatov and Pearl (1929), studying longevity of *Drosophila* under different temperatures, observed that females were longer lived than the males in all series of experiments. Gonzalez (1923) attempted to localize the factors controlling the duration of life of *Drosophila.* He also studied the effects of food and temperature of its life span. Bowen (1936) found that the male outlived the female in *Trichogramma,* a parasite on the oriental fruit moth, and Menusan (1936) demonstrated that temperature and humidity have marked effects on duration and viability of different stages of bean weevil, *Bruchus obtectus.*

Lilleland (1938) used *Drosophila pseudoobscura* in experiments dealing with the duration of life in the absence of food and made comparisons at different temperatures, humidities, and population densities. Greiff (1940) investigated the duration of life of wild-type *Drosophila melanogaster* and its ebony mutant in the absence of food and water. He found that the mean duration of life of the ebony mutant exceeded that of the wild-type in a statistically significant manner. The mean length of life of the wild-type male was significantly greater than the mean length of life of the wild-type female, but the difference between the mean duration of life of the ebony female and that of the ebony male was non-significant.

Up to the present time the problem of longevity in *Habrobracon juglandis* (Ashmead) has been only occasionally and insufficiently attacked by other investigators. Doten (1911) worked with small samples of *Habrobracon* under normal laboratory conditions with temperatures ranging between 70° and 80° F. He reported that females if fed on honey water lived an undetermined number of months, and that they outlived the males. If not fed at all the females died in from 11 to 15 days. Males when fed were kept alive for an indefinite time, but if not fed, they died in a week or ten days. Doten concluded by emphasizing the necessity of controlling the conditions under which the experiments are performed in order to draw valid conclusions.

Harries (1937) determined the approximate duration of the developmental stages of *Microbracon hebetor,* which is synonymous with *Habrobracon juglandis,* but he did not report any investigation of the duration of life of the imago. Extensive work has been done on *Habrobracon* by Dr. P. W. Whiting, of the University of Pennsylvania, and by his coworkers who have investigated many phases, but there is no evidence in literature that any of them have studied the life span of this insect.

In the following investigation on longevity of the parasitic wasp, *Habrobracon juglandis,* a comparative study was made of the mean duration of life of the wild-type and of the double mutant short-wing, white-eye type, which will be referred to in this report as the double mutant.

The writer is deeply indebted to Dr. E. S. McDonough, of the Department of Biology of Marquette University, under whose kind direction the present investigation was carried out, and wishes to express sincere appreciation for his advisory assistance and helpful criticism during the progress of this study; to Rev. R. H. Reis, S.J., head of the Biology Department of Marquette University, for help and encouragement; and to Dr. P. W. Whiting, of the University of Pennsylvania, for valuable criticism, literature and other help.

MATERIALS AND METHODS

All the experiments in this investigation were carried out under constant environmental conditions. The cultures were incubated at about 30° C. which, according to Whiting (1921) and Maercks (1933), is the optimum temperature for *Habrobracon*. The humidity in the incubator was controlled by employing a saturated solution of ammonium chloride which has been reported by Obermiller (1924) and in the International Critical Tables (1926) to maintain a humidity of 79.5 per cent at 30° C. Water was added daily to the solution, or when necessary, to maintain a certain level which had been indicated on the outside of the beaker.

The two stocks of *Habrobracon* as well as the host caterpillars were obtained from Dr. P. W. Whiting, and the technique used in handling the insects was that described by Whiting (1921, 1937).

A pair of wasps was selected and placed with four full-grown host caterpillars in a shell vial 20 × 70 mm. and incubated. After mating, the female stings the caterpillars and sucks juice from the punctures. The males must be fed honey-water as they do not feed on caterpillars. After the caterpillar has become flaccid, the female deposits her eggs on it, usually on the under side. In about four days, tiny maggot-like larvae may be observed clinging to the integument of the host caterpillars. The adult wasps are now transferred to a new vial with fresh caterpillars. Growth of the *Habrobracon* larvae is rapid and soon white cocoons appear, and the host caterpillars are reduced to shrivelled remnants. In ten days from the time the pair was set, eclosion begins. From four to seven wasps were obtained from each host caterpillar.

As soon as the adults of *Habrobracon* emerged, they were transferred to shell vials. A population density of four males or four females per vial was maintained throughout the investigation. As was pointed out by Pearl and Parker (1922):

> Putting all data together we have here indisputable evidence that the density of population is a significant factor in influencing the duration of life.

Wasps that seemed abnormal or undernourished were not used for experimentation, but were discarded, and the experimental stock was not used for breeding, but was segregated before mating. The vials containing the test stock were labeled and placed flat in racks in the incubator. All stock was fed honey-water every other day. This honey-water consisted of three parts of honey to two parts of water which was mixed and heated slightly to prevent fermentation and to insure the possibility that all insects received the same concentration of honey. Routine feeding was accomplished by using a small medicine dropper with which a small drop of food was placed in the top-arch of the vials which housed experimental *Habrobracon*. This, according to Doten (1911), reduced the entanglement of insects in honey-water. Losses due to entanglement were also cut down to a minimum by removing remains of old honey before administering a new droplet. This precaution also serves as a safeguard against mold and other contaminations.

Each morning at the same hour the test stock was examined and records were made of those that had died. Care was taken that no vials were out of the incubator for more than fifteen minutes during routine feeding and checking. To test the validity of results, breeding was continued until four or five runs of experiments had been made of both males and females of the two stocks, yielding 1,180 wild-type wasps, and 1,366 double mutant wasps. Thus a total of 2,546 wasps was tested.

RESULTS

Table I presents the frequency distribution of males and females, respectively, of each of the two stocks of *Habrobracon*.[1] The class centers of the various groups are shown, together with the number of wasps that died in the respective five-day intervals. Table II gives the chief biometric constants[2] derived from this investigation.

The mean duration of life of the wild-type female was found to exceed that of the wild-type male by 5.157 days

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[1] A daily record was kept of the number that died, but in order to conserve space only the frequency table is included here.

[2] The short method described in "Principles of Genetics," by Sinnott and Dunn, p. 140, was used in computing the various constants.

TABLE I

FREQUENCY DISTRIBUTION OF 1,180 WILD-TYPE AND 1,366 DOUBLE MUTANT WASPS

Class center in days	Number of Habrobracon that died in the 5-day period			
	Wild-type		Double mutant	
	Males	Females	Males	Females
4	12	15	18	16
9	40	26	80	30
14	81	26	176	74
19	142	41	214	89
24	165	62	193	145
29	129	119	81	85
34	58	75	41	58
39	52	44	13	24
44	21	37	3	13
49	....	24		13
54		11		
	700	480	819	547
Total	1,180		1,366	

A daily record was kept of the number that died, but in order to conserve space only the frequency table is included here.

TABLE II

CHIEF BIOMETRIC CONSTANTS

	Mean duration of life (days)	Standard deviation (days)
Wild-type male	24.093 ± 0.334	8.825 ± 0.236
Wild-type female	29.250 ± 0.517	11.335 ± 0.366
Double mutant male	19.946 ± 0.259	7.410 ± 0.183
Double mutant female	23.991 ± 0.407	9.595 ± 0.288

The short method described in "Principles of Genetics," by Sinnott and Dunn, p. 140, was used in computing the various constants.

under the conditions of this experiment. By dividing this difference by its own standard error, it was found that this difference was statistically significant.

The mean duration of life of the double mutant female exceeded that of the double mutant male by 4.045 days. This difference was statistically significant.

The mean duration of life of the wild-type male exceeded that of the double mutant male by 4.147 days, and this difference was found to be statistically significant.

The mean duration of life of the wild-type female exceeded that of the double mutant female by 5.259 days, and this difference also was found to be statistically significant.

DISCUSSION OF RESULTS

In this investigation the mean duration of life of the wild-type wasp was significantly longer than that of the double mutant wasp. In endeavoring to explain this difference it is important to consider the part that food and environment play in the life of an animal.

It is a well known fact that, aside from temperature, an adequate food supply is essential to normal life, and that the nature of the food affects the life span. Pearl and Parker (1924) advanced the idea that the inherent potentiality of the organism in respect of longevity is determined by its individual, inborn physico-chemical organization, and that the realization or expression of the potentiality of longevity is a function of the environment, for this environment determines in considerable part the rate at which the vital resources are used up. In considering the physiology of animal metamorphosis, Kopec (1924) remarked:

> It seems to me to be unquestionable that the duration of life depends on the character of metabolism, in other words, that natural death is a function of metabolism.

This is further substantiated by Pearl and Parker (1924):

> Normally the living organ gains its energy for the conduct of life, and material for the repair, within limits, of the wastage of its tissues in the business of living, by the process of taking food. In other words the regular rewinding of the vital clock is accomplished by feeding. If the renewal or rewinding process is defective in any particular, the result will be to shorten life below what would have been attained under more perfect metabolic functioning.

The normal life span of an organism seems to be determined by the production of a substance leading to old age and natural death, or by the destruction of a substance which normally prevents old age and natural death. According to Ruzicka (1929) longevity also depends on the rate of gradual condensation of biocolloids which underlie the process of aging. He suggests:

> The gene of duration of life is not preformed but it arises by continual changes of biocolloids provoked by external influence and ends by condensation rendering metabolism impossible, thus causing death.

After considering the above, and keeping in mind that the technique used insured constant environmental conditions throughout all the experiments, it becomes evident that environmental factors are not the basis of the difference in longevity. Since both strains were reared at the same time, the difference noted is definitely believed to be hereditary. In these investigations the double mutant short wing, white-eye wasps were shorter lived. The mutant genes apparently affect the summation of physiological processes under the conditions of these experiments so that the mean length of life is shortened. It might be suggested then that the mutant genes have as a part of their somatic expression a reduced duration of life as compared with the wild-type, under the conditions of these investigations. Referring to the work done by Gonzalez (1923) on longevity, Jennings (1939) remarks:

> Every gene (with seemingly few exceptions) plays an essential role in the life and development of every cell of the body. It is not surprising, therefore, that changing a single gene may so alter the cellular processes as to change the length of life.

Longer duration of life in the female over that of the male is also explained on the basis of heredity rather than environment, for it might be regarded as a constitutive element of females. To the female is attributed a lower metabolic rate; consequently, in females there is a slower rate of the gradual condensation of biocolloids, and also a retardation of the processes which render metabolism impossible. As a result of this retardation there is a slower production of a substance leading to old age and natural death.

In addition to the physiological processes which lengthen or shorten the life span of insects, it must be recognized that we have at work natural selection which regulates these physiological processes in some way, thus prolonging the life of the female to insure oviposition which is an advantage to the race and curtailing useless existence of males which become sterile after a few weeks.

It must be borne in mind that the results obtained under the conditions of these investigations may be altered or perhaps even reversed under other conditions, and it would be interesting to investigate whether the results were comparable to the above if the adults of *Habrobracon* were subjected to a starvation diet. It is not improbable that cohabitation of males and females would decrease the mean duration of life of both sexes.

SUMMARY

The results of the investigations of duration of life of 1,180 wild-type and 1,366 double mutant short-wing, white-eye individuals of the parasitic wasp, *Habrobracon juglandis* (Ashmead), under constant conditions of about 30° C. temperature, about 79.5 per cent relative humidity, with a population density of four males or four females, and a food diet of honey-water, were as follows:

(1) The mean duration of life of wild-type male was 24.093 ± 0.334 days, and that of the wild-type female was 29.250 ± 0.517 days. The mean duration of life of the female exceeded that of the male by 5.157 days. The difference was statistically significant.

(2) The mean duration of life of the double mutant male was 19.946 ± 0.259 days as compared with that of the double mutant female which was 23.991 ± 0.407 days. The mean duration of life of the female exceeded that of the male by 4.045 days. The difference was significant.

(3) The mean duration of life of the wild type male exceeded that of the double mutant male by 4.147 days. The difference was statistically significant.

(4) The mean duration of life of the wild -type female exceeded that of the double mutant female by 5.259 days. The difference was statistically significant.

(5) The mean duration of life of the wild-type, sexes combined, was 26.186 days, and the mean duration of life of the double mutant, sexes combined, was 21.566 days. The difference in their mean duration was 4.620 days, and this difference, too, was statistically significant.

LITERATURE CITED

Alpatov, W. W. and Raymond Pearl
1929. Am. Nat., 63: 37-67.
Bowen, M. F.
1936. *Ent. Soc. Amer.*, 29: 119-125.

Doten, Samuel B.
1911. *Tech. Bul.* No. 78, Agricultural Exp. Station, University of Nevada, Reno.
Gonzalez, Maria Bienvenida
1923. Am. Nat., 57: 289-326.
Greiff, Donald
1940. Am. Nat., 74: (753) 363-376.
Harries, F. H.
1937. *Ohio Jour. Sci.,* Columbus, 37: 165-171.
Jennings, H. S.
1939. "Senescence and Death in Protozoa and Invertebrates," "Problems in Aging." Ed. by E. V. Cowdry.
Kopec, Stefen
1924. *Biol. Bul.,* 46: 1.
Loeb, Jacques, and J. H. Northrop
1917. *Jour. Biol. Chem.,* 32: 103-131.
1916. *Proc. Nat. Acad. Sci.,* 2: 456-457.
Lutz, Frank E.
1915. *Bul. Am. Mus. Nat. Hist.,* 34: 605-624.
Maercks, Hans
1933. *Ait. Biol. Reichsanst. Lane U. Forstw.* Berlin, Dohlem, 20: (3) 347-389.
Menusan, J., Jr.
1936. *Ent. Soc. Amer.,* 29: 278-288.
Northrop, John H.
1917. *Jour. Biol. Chem.,* 32: 123-126.
Obermiller, J.
1924. *Zeitschr. f. Physik. Chem.,* 109: 145.
Pearl, R., and Sylvia Parker
1922. Am. Nat., 56.
1924. Am. Nat., 58.
Rau, Philip
1912. *Jour. Exper. Zool.,* 12: 179-204.
1914. *Trans. Acad. Sci. St. Louis,* 23: 1-78.
Ruzicka, Vlad
1929. Congres. Internat. Zool. a Budapest 1927 Pt. 1. 652-657.
Whiting, P. W.
1921. *Jour. Heredity,* 12: 255, 262.
1937. "Culture Methods for Invertebrate Animals." Ed. by Comstock.

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SIX MUTATIONS AFFECTING COAT COLOR IN RANCH-BRED MINK [1]

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TWELVE mutant genes affecting coat color in the ranch-bred mink (*Mustela vison*, Peale and Beauvois) are now recognized. The well-known and commercially valuable color phases resulting from the substitution of six of these for their non-mutant alleles have been described by Smith *et al.* (1941), Shackelford (1941) and Castle and Moore (1946). The last mentioned authors have called attention to a few color phases resulting from combinations of certain of the mutant genes.

Smith *et al.* have proposed p as the symbol for the mutant gene for platinum, b for pastel and S for black cross. Castle and Moore have assigned c^{a} to the gene for near albinism, ip for imperial platinum and F for blufrost. Although Castle (1946) later suggested S rather than F as the correct symbolization of the gene for blufrost by the rule of priority, by this same rule F should be considered the proper symbol because of the following circumstances: The color phase known at present as black cross was originally designated silver by Smith *et al.* and the mutant gene symbolized by *S:* Castle incorrectly states that silver " came to be known later by the popular name silver sable, and, more recently, blufrost." Therefore, since Smith *et al.* symbolized the gene for black cross and not the one for blufrost, Castle and Moore's original use of *F* is valid.

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[1] Cooperative experiments between the Wisconsin Conservation Department's Experimental Game and Fur Farm, Poynette, and the Department of Genetics (Paper No. 402), University of Wisconsin. Supported in part by Wisconsin Agricultural Experiment Station, Project 614, Fur Farm Research. Published with the approval of the Director of the station.

The known mutant genes affecting coat color in American ranch-bred mink and foxes were enumerated and briefly discussed in a review paper given before the Eighth International Congress of Genetics by this writer. The six genes in the mink which had already been symbolized by other workers as indicated above were included in the paper along with the six to be considered here.

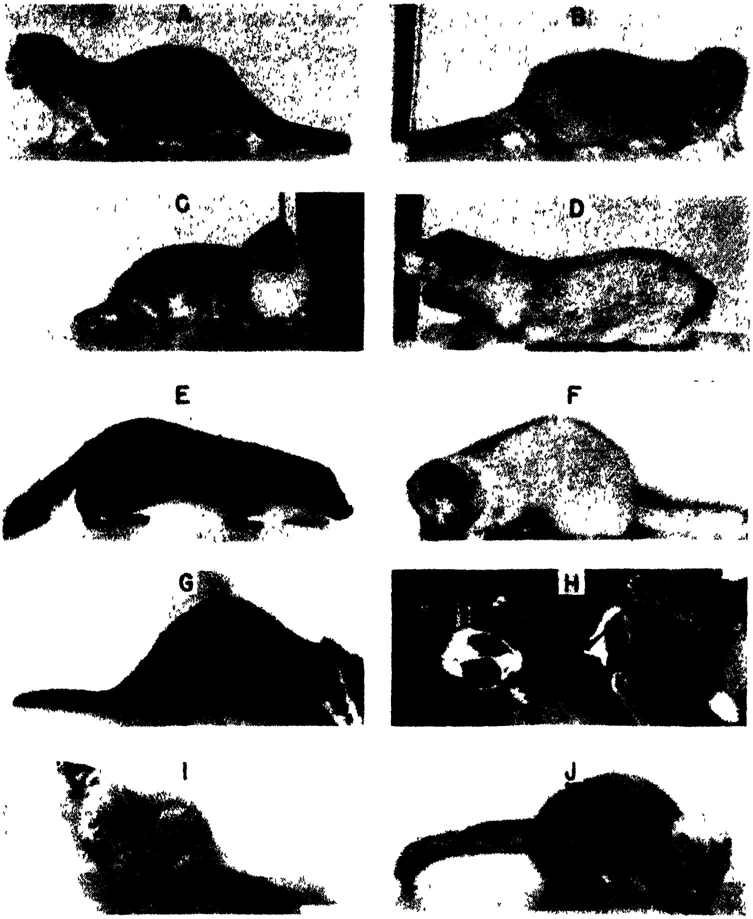
It is not uncommon to encounter considerable confusion when working with mutant color phases in the mink. The same basic genotype may have been known at various times since its recognition by four or five popular names. On the other hand, phenotypically similar but genetically different color phases have often borne the same name at some time in their development. In common with some other mammals, several of the mutant color phases in the mink seem to have " cropped up " at geographically widely separated places. These conditions make it difficult if not impossible to accredit correctly any given person as the " originator " of a certain color phase.

The treatment of the color phases to be considered in this paper will be based on the breeding results from our experimental herd in so far as possible, liberally supplemented with records taken from commercial breeders either for additional data or to supply data which are entirely lacking at this time. Speculation as to the probable value of any of these mutant genes for producing " new " color phases of commercial value will be left to those better qualified to consider such matters.

DOMINANT COLOR PHASES

Royal Silver

Chokeř, royal silvered and simply royal silver are some of the names used to designate a pattern that has been known to breeders for several years as one of the "*dominant*" color phases. It differs from the ordinary dark mink (wild type) mainly in the extension of the ventral white spotting to include the entire chin, throat, breast, belly and sometimes the feet and tail tip (Figure 1, A and B). Often



EXPLANATION OF PLATE

FIG. 1. Some color phases of the ranch-bred mink. A. Royal Silver (s^Rs). B. Glacierblu (s^Rspp). C–D. Blucross (F/s^Rs). E. Ebony ($Ebcb$). F. Black cross heterozygous for Royal Silver (Ss^R). G–H. Colmira ($Cmcm$). I–J. Goofus (oo).

completely white guard hairs are scattered over the dorsal parts and the ventral white regularly extends up to the base of the ears. As with all the color phases in the mink, especially patterns, royal silvers differ considerably in appearance one from the other presumably as a result of modifying factors. Some of the least pigmented individuals in the royal silver group might occasionally be confused with the most heavily pigmented members of the black cross color phase.

Some breeders believe a darkening of the color in the guard hairs not affected by the white spotting to be regularly associated with this color phase. The breeding results in our herd, however, indicate that the darker color is dependent upon the residual inheritance rather than the mutant gene for the royal silver pattern.

Royal silver was introduced into our herd via two males presented by Dr. S. S. Osborn, of Waterville, Minnesota, in 1946. The pedigree of these males was unknown, but breeding results indicate that they are homozygous for the gene (s^R) responsible for the royal silver pattern (Table I A). Seven royal silver (s^Rs) offspring of these males in matings to non-royal silver (ss) produced 11 litters with a total of 23 royal silver to 21 non-royal silver young (Table I B). The two original royal silver males (2.1 and 2.2 in Table I A) are phenotypically similar to their offspring which are known by pedigree and breeding results to be heterozygous for s^R.

A female kit that in all respects is prenotypically identical with the royal silver color phase was born of natural dark (wild type) parents in 1945 on the Fromm Brothers, Nieman and Company ranch at Thiensville, Wisconsin. Since the royal silver had not previously been observed on this ranch, it seems probable the color phase of the kit resulted from a mutation at the s locus. The breeding history of this female and her royal silver offspring, both male and female, is similar to that of the royal silvers (s^Rs) in our herd.

TABLE I

BREEDING RESULTS OF THE ROYAL SILVER COLOR PHASE

	Type Mating	Offspring								Number litters	Average litter size
		Royal Silver		Non-Royal Silver							
		♂	♀	♂	♀						
	Royal Silver ♂ 2.2 × Non-Royal Silver ♀♀	24	16	0	0					9	4.4
A	Total	40		0							
	Royal Silver ♂ 2.1 × Non-Royal Silver ♀♀	13	12	0	0					6	4.2
	Total	25		0							
	Royal Silver ♂♂ × Non-Royal Silver ♀♀	6	4	7	3					6	3.3
B	Non-Royal Silver ♂♂ × Royal Silver ♀♀	8	5	5	6					5	4.8
	Observed	23		21							
	Expected (1:1)	22		22							
		Black Cross		Royal Silver		Dark					
		♂	♀	♂	♀	♂	♀				
C	Black Cross ♂ × Royal Silver ♀♀	4	5	1	1	1	0			4	3 0
	Observed	9		2		1					
	Expected (2:1:1)	6		3		3					
	Black Cross ♂ 28 × Dark (Wild Type) ♀♀	3	3	6	2	0	0			4	3.5
D	Dark (Wild Type) ♂♂ × Black Cross ♀ 109	1	1	3	1	0	0			2	3.0
	Observed	8		12		0					
	Expected (1:1)	10		10		0					
	(2:1:1)	10		5		5					
		Blucross		Royal Silver		Blufrost		Dark			
	Dark (Wild Type) ♂♂ × Blucross ♀♀	16		10		11		15		12	4.3
	Expected (1:1:1:1)	18.25		18.25		13.25		18.25			
		♂	♀	♂	♀	♂	♀	♂	♀		
E *	Blucross ♂ 2 × Dark (Wild Type) ♀♀	2	0	0	1	2	0	1	0	2	3.0
	Dark (Wild Type) ♂♂ × Blucross ♀ 4	0	1	1	0	0	1	0	1	2	2.0
	Observed	3		2		3		2			
	Expected (1:1:1:1)	2.5		2.5		2.5		2.5			

* Data obtained from Dr. S. S. Osborn, Waterville, Minnesota.

Castle and Moore (1946) reported " an imperfectly tested dominant mutation " which produced a color phase they have termed "Blackout." This may be the same mutant gene (s^R) with which we are dealing here. The picture of the heterozygous individual shown in their paper is a good representation of either the heterozygous or homozygous royal silver mink in our herd, although none of our royal silvers have approached the appearance of the one they have called a "Homozygous Blackout individual." Further, these workers have suggested that "Possibly it (Blackout) is an allele of Kohinur." As will be seen from the discussion to follow, the evidence from our breeding experiments supports the hypothesis that the mutant genes for royal silver (s^R) and black cross (S) are alleles.

That the black cross color phase expresses itself to the exclusion of the royal silver was indicated by the results of black cross × royal silver matings in 1947 (Table I C). Approximately twice as many black cross kits occurred as would be expected unless about one-half of them were also heterozygous for the gene for royal silver. Two out of three of the phenotypically black cross individuals (one shown in Figure 1 F) from this type mating which were test mated to darks (wild type) produced among their offspring typical royal silver kits, thus proving them to be heterozygous for the gene for the royal silver pattern. The breeding results of these two black cross mink for 1948 and 1949 are shown in Table I D. Together they have produced 8 black cross and 12 royal silver kits when mated to dark (wild type) mink.

One kit representing the dark class would have been sufficient to demonstrate that S and s^R are not alleles. Or, if a black cross individual from this type mating produced royal silver offspring when mated to wild type, this would also demonstrate non-allelism; one such black cross has been tested, and has thrown black cross and dark kits only. If it is assumed that S and s^R are non-allelic, then the expected ratio in Table I D would be 10 black cross to 5 royal silver to 5 dark kits. The observed ratio of 8 black cross to 12 royal silvers is a very poor fit to the expected 2:1:1 ratio ($X^2 = 15.2$, $P = .0005$). On the assumption that S and s^R are alleles, the observed ratio is a good fit to the expected 1 : 1. Thus the evidence indicates that S and s^R form an allelic series, with dominance in the direction $S \rightarrow s^R \rightarrow s$.

Osborn (1946) has mentioned a color phase which he calls blucross from blufrost (*Ffss*) × royal silver (ffs^Rs) matings. Although some individuals of this color phase (Ffs^Rs) differ slightly in appearance from the ordinary black cross (*Ss*), most of them are so similar to black cross that they would be classed among the commonly observed variations of black cross by those uninformed as to their genotype (compare C and D with F in Figure 1). Confusion has arisen as a result of using this name for the genotype Ffs^Rs. Blucross as a color phase name is more often applied to the genotype *Sspp*, that is, the " black cross pattern on platinum " combination. Osborn's usage of blucross for the genotype Ffs^Rs will be followed in this paper.

Twelve litters from blucross (Ffs^Rs) × dark (*ffss*) matings as observed and recorded on the Osborn ranch in June, 1946, are shown in Table I E. The presence of either the blucross (Ffs^Rs) class or the dark class (*ffss*) is sufficient evidence that s^R and F are not alleles. Further, the four classes appeared in approximately equal numbers, indicating that these two genes are borne on different chromosomes ($X^2 = 2.0$, $P > .55$), or if on the same chromosome, are not closely linked.

A blucross male and female were produced from a blufrost × royal silver mating in our herd in 1946. The two blucrosses are shown in C and D of Figure 1. The breeding results of these two mink in the 1947 and 1948 seasons are shown in Table I E, confirming the data collected on the Osborn ranch in 1946.

The color phases resulting from s^R in combination with *p*, *b* and *Cm* have been observed. Mink of the genotype s^Rspp are royal silver patterned platinums sometimes called glacierblu by breeders (Figure 1 B). s^Rsbb is a pastel with the royal silver pattern. In a mink of the genotype s^RsCmcm, the separate effects of both s^R and Cm are evident with no indication of interaction between them.

Colmira

Osborn (1946) has called attention to another type of white spotting in the mink and has designated it colmira (Figure 1 G and H). In common with royal silver, colmira has been known to mink breeders for some years as one of the several "dominant" color phases. So far as is known the gene (*Cm*) for colmira spotting has been observed only in the heterozygous condition, presumably because of a lack of matings designed to test its affects when homozygous.

Like the royal silver pattern, the ventral white spotting in the colmira color phase includes the chin, throat, breast, belly, sometimes the feet and tail tip (Figure 1 H), and often completely white guard hairs are sprinkled over the dorsal parts. Unlike royal silver or any of the other white spotting patterns in the mink, colmira spotting is rarely symmetrical on the head and neck. The white in these areas may range from a narrow blaze up the nose and forehead (Figure 1 G), to a large blaze joining with a complete collar about the neck including considerable portions of the shoulders; in the latter extreme, the only pigmented areas about the face may be a colored spot around each ear (Figure 1 H) rarely of the same size and sometimes around only one ear. Colmiras with much white about the face and head are occasionally called "panda" by mink breeders. Along with the asymmetry of the white spotting, the underfur in the colmira is characteristically lighter in color than in the natural dark. The brief description and picture in Castle and Moore's 1946 paper indicates that their "Baldy" is at least phenotypically similar to the color phase here termed colmira.

Colmira matings in our herd are limited to a single male (Figure 1 H) presented by Dr. Osborn in 1947, and two of his male offspring. This male, M38, proved to be heterozygous for *Cm* as shown in Table II. Four litters produced by his colmira offspring in matings to non-colmira mink gave 7 colmira to 10 non-colmira kits, a close approximation to the expected 1 : 1 ratio ($X^2 = .52$, $P = .50$).

TABLE II

COLMIRA × NON-COLMIRA MATINGS

	Type Mating	Offspring				Number litters	Average litter size
		Colmira		Non-Colmira			
		♂	♀	♂	♀		
A	Colmira ♂ M38 × Non-Colmira ♀♀	9	3	2	1	4	3.8
B	Colmira ♂♂ × Non-Colmira ♀♀	3	4	4	6	4	4.3
	Total	19		13			
	Expected (1:1)	16		16			

A mating of colmira (*Cmcmff*) × blufrost (*cmcmFf*) resulted in a litter containing 3 blufrost-colmira kits, 2 blufrosts and 1 colmira. From the appearance of the three mink showing the effects of both *Cm* and *F*, these two mutant genes appear to exert their separate effects with no indication of interaction in the combination *CmcmFf*. A blufrost-colmira (*CmcmFf*) male from this litter mated to dark (*cmcmff*) females has produced two litters which total 1 dark, 1 blufrost and 2 colmira kits. The appearance of the single dark individual is sufficient evidence that *Cm* and *F* are not alleles. A male mink from a black cross (*Sscmcm*) × colmira (*ssCmcm*) mating which showed the effects of both *S* and *Cm* was obtained from Dr. Osborn in 1948. This colmira-black cross male (*SsCmcm*) gave two litters when mated to dark (wild type) females; one contained 2 colmira, 2 black cross and 1 dark, and the other was composed of 1 colmira, 1 black cross and 1 dark. The presence of the dark kits (*sscmcm*) indicated that *S* and *Cm* are non-allelic.

Cmcmbb is the most commonly observed combination of colmira with any of the other color phases and is known among breeders as the " red-eyed blue taupe " color phase. This combination produces a white-spotted pastel with light underfur, no individuals with other than " red " eyes (darker than the eyes of the albino) having been observed. Mink of the genotype *Cmcmpp* are colmira patterned platinums. The $s^{R}sCmcm$ genotype has already been considered.

The original colmira male M38 and all of his 19 direct or indirect colmira descendants show a remarkable lack of sensitivity to disturbances when sleeping. This does not appear to be due to an inability to hear, since the colmira, when awake, responds to ordinary sounds as well as other mink do. The degree to which colmira mink can resist disturbances and remain asleep is best demonstrated by approaching a nest box in which a mixed litter is sleeping. A touch of the nest box or at most raising the top is sufficient to send all non-colmira kits scurrying, but their litter mate colmiras usually remain asleep and can be touched or even gently picked up and replaced without awakening. Once awake, however, the colmira is as active as other mink.

Another behavior pattern associated with the colmira color phase is an apparent lack of coordination which in certain of its aspects is somewhat suggestive of "screw neck" (Shackelford and Cole, 1947) in the brown-eyed pastel, but in other aspects is quite different. Nearly all colmira patterned individuals throw the head over the back at times as do some pastels, but unlike the pastels they do not tilt the head to either one side or the other. A few colmira individuals run in small circles, but again unlike circling pastels do not necessarily have to be disturbed in order to exhibit circling. In general colmiras seem to be less upset by disturbances than do pastels, and sometimes actually appear to enjoy practicing the circling antics.

Three of these 20 colmiras, all males, are subject to convulsive seizures. Unusual noises or disturbances do not seem to be involved in precipitating the seizure. The affected mink will be running about in its cage like a normal individual, then suddenly fall prostrate, and a period of general twitching and jerking with foaming at the mouth follows and may last 20 to 30 minutes. When the mink has recovered it appears to be quite normal and shows no ill effects of the experience.

Ebony

A coat color that has been recognized by breeders for some years as belonging to the " dominant " group is called ebony or ebonyblu, and in contrast to the other color phases seems never to have been known by other names. The underfur of the ebony is considerably lighter than that of the natural dark mink, ranging from near white to blue gray. The color of the guard hairs and the ventral white spotting remain the same as in the natural dark. In a segregating litter, the very young ebony kits can usually be distinguished from their non-ebony litter mates by the lighter appearance, particularly on the nose, face and feet.

Matings involving the ebony color phase in our herd are limited to two individuals, a male and a female, which are heterozygous for the gene (*Eb*) for ebony since they have produced both ebony and non-ebony kits when mated to non-ebony mink. The complete breeding data for these two mink can not be evaluated at this writing because of our inability to classify phenotypically some individuals having other dominant genes which may be obscuring the effects of *Eb*.

TABLE III *

BREEDING RESULTS OF THE EBONY COLOR PHASE

	Type Mating	Offspring				Number litters	Average litter size
		Ebony	Non-Ebony				
A	Ebony ♂♂ & ♀♀ × Non-Ebony	87	102			46	4.1
	Expected (1:1)	94.5	94.5				
B	Ebony (EbebBb) ♂♂ & ♀♀ × Pastel (ebebbb)	Ebony (EbebBb)	Dark (ebebBb)	Palomino (Ebebbb)	Pastel (ebebbb)		
		22	14	7	37	15	5.3
	Expected (1:1:1:1)	20	20	20	20		

* Data obtained from Christensen Brothers Minkery, Cambridge, Wisconsin.

The data shown in Table III were taken from the 1948 breeding records of the Christensen Brothers Minkery, Cambridge, Wisconsin. Ebony (derived from ebony × natural dark matings) × natural dark matings gave 102 natural dark to 87 ebony kits, a ratio which does not differ significantly from 1 : 1 ($X^2 = 1.2$, $P > .27$).

Within certain limits the most desirable pelt in any self-colored mink is the one showing greatest contrast between the guard hair and the underfur. In order to enhance the "two tone" effect of the pastel (*bb*), several breeders have made the *Ebebbb* combination producing a color phase called by some palomino. This combination has been achieved by making *EbebBB* × *ebebbb* matings, followed by *EbebBb* × *ebebbb* matings. Tale III B shows the results of the latter type mating in 1948 on the Christensen Brothers' ranch.

On the assumption that *Eb* and *b* are on different chromosomes the four expected color phases should approximate a 1:1:1:1 ratio. The observed 22 ebony (*EbebBb*) : 14 dark (*ebebBb*) : 7 palomino (*Ebebbb*) : 37 pastel (*ebebbb*) is a very poor fit to the expected ratio ($X^2 = 20.0$, $P = .0002$). If it be assumed that *Eb* and *b* are on the same chromosome, then *Eb* and *b* are located approximately 26 crossover units apart since the non-crossover classes (ebony and pastel) comprise 74 per cent. of the total from this type mating and the crossover classes (palomino and dark) 26 per cent. So far as is known these data furnish the first evidence of linkage in the mink.

Blufrost (*Ffebeb*) × ebony (*ffEbeb*) matings have been made in our experimental herd as well as on a number of commercial ranches. *Eb* and *F* do not appear to interact in the combination *EbebFf*. Mink of this genotype closely resemble the blufrost color phase, but can usually be distinguished from it by the lighter tone of the underfur along with a lighter brown patch over each eye somewhat resembling an eyebrow, and the light brown margins of the ears.

Several breeders have attempted to obtain the ebony-platinum combination (*Ebebpp*). Either this genotype has not been produced, or its phenotype is similar to platinum since only ebony, dark and platinum kits have been observed among more than 150 offspring from *EbebPp* × *ebebpp* matings. The mink homozygous for *Eb* has either not been produced or else not recognized.

RECESSIVE COLOR PHASES

Aleutian

Langenfeld (1947) has given an account of the occurrence and early breeding history of a color phase originally called Waris blue, but now most commonly known as aleutian. This color phase results from a third mutation that dilutes the natural dark coat color to a blue gray. Although the overall appearance of the aleutian suggests the platinum (*pp*) and imperial platinum (*ipip*) phases which are phenotypically similar, it is darker and more nearly approaches a steel gray color. The smoother or softer tone of the aleutian in comparison with platinum or imperial platinum probably is a result of the distribution of the pigment granules in the hair; in the platinum (Shackelford, 1948) the dark brown pigment granules are for the most part in large clumps, while in the aleutian what appears to be a reduced quantity of pigment is evenly distributed within the hair. The eyes of some aleutians are red when seen at certain angles.

All breeders of this color phase are agreed that the gene (*al*) for aleutian is recessive to its non-mutant allele since dark (wild type) offspring only are produced from aleutian × dark matings, back cross matings of *Alal* × *alal* give aleutian and dark kits in approximately equal numbers, and aleutian × aleutian matings produce only aleutian kits. We have had only two matings involving the aleutian color phase in our herd. In the 1948 breeding season Associated Fur Farms mated two of our dark (wild type) females to aleutian males. The 13 kits from these matings were all dark (Table IV A). Test matings in 1947 and 1948 at Associated Fur Farms of aleutian (*alalPP*) × platinum (*AlAlpp*) and aleutian (*alalIpIp*) × imperial platinum (*AlAlipip*) gave only wild type offspring as would be expected if *al* represented a mutation at a different locus from *p* and *ip* (Table IV B and C).

Several color phases resulting from the combination of *al* with other mutant genes are popular among breeders. The

TABLE IV

BREEDING RESULTS OF THE ALEUTIAN COLOR PHASE

	Type Mating	Offspring				Number litters	Average litter size
		Aleutian		Dark			
		♂	♀	♂	♀		
A	Aleutian ♂♂ × Dark ♀♀	0	0	10	3	2	6.5
B	* Aleutian ♂♂ × Platinum ♀♀	0	0	15	10	6	4.2
C	* Aleutian ♂♂ × Imperial Platinum ♀♀	0	0	17	17	9	3.8
D	** Dark (AlalPp) × Dark (AlalPp)	Dark	Platinum	Aleutian	Sapphire		
		53	17	24	8		
	Expected (9:3:3:1)	57.4	19.1	19.1	6.4		

* Obtained from Associated Fur Farms, New Holstein, Wisconsin.

** Obtained from Messrs. Edward Langenfeld, Boch and Mohr, Andrew Sturgeon and Don Lee.

sapphire color phase is the most nearly "blue" of any of the mutant color phases and for breeding purposes is the most sought type in the history of mink breeding. The data presented in Table IV D show the combined results of *AlalPp* × *AlalPp* matings on four Wisconsin ranches. The observed 53 dark : 17 platinum : 24 aleutian : 8 sapphire is a good fit to the 9:3:3:1 ratio, suggesting that *al* and *p* are borne on different chromosomes ($X^2 = 2.23$, $P > .5$). Sapphires of the two genotypes *alalipip* and *alalpp* are phenotypically indistinguishable and both occasionally have red eyes.

The *Ffalal* genotype is known as the arctic or breath-of-spring aleutian and shows the pattern effect of *F* superimposed on the steel gray of the aleutian. Eric is the aleutian-brown-eyed pastel combination, *alalbb*. This genotype always has pink eyes, darker than the eyes of the albino mink. The pleasing soft tone of the Eric pelage is difficult to describe, but most nearly approaches a bluish fawn color. Individuals of this color phase are often characterized by an irregular splotching, the spots being a few shades further toward the steel gray of the aleutian than the bluish fawn background; factors involved in the splotching are yet to be analyzed.

Green-Eyed Pastel

The green-eyed pastel or golden cross color phase first appeared on the ranch of Mr. Wayne Jones, of Ontario, Wisconsin, in 1941, and so far as is known all green-eyed pastels are descendants of mink from the Jones ranch. The first male and female of this color phase were born along with natural dark kits in litters of two natural dark females bred to the same natural dark male. The male and the females were not known to be related, the two females coming of stock purchased from one ranch and the male from another.

Some green-eyed pastels are considerably lighter in coat color than the average brown-eyed pastel (*bb*). The lightest individuals have lighter brown markings on the underparts which extend upward along the sides of the body, the sides of the neck, and the face; these markings are apparently responsible for the name "golden cross." In the darker individuals the pattern can not be detected, and they appear similar to the brown-eyed pastels. This slight pattern effect may be the result of modifying factors rather than a characteristic effect of the mutant gene per se.

The most characteristic feature of the green-eyed pastel that will usually differentiate it from the brown-eyed pastel phenotypically is the eye color; in most animals the eyes are a luminous green, especially when the mink is in a shaded place looking at the observer. At certain angles in the bright sunlight the eyes appear luminous red, and at other angles are green as seen in the shade. The eye color is occasionally light brown similar to that of the lightest of the brown-eyed pastels; the darker eyed individuals invariably have the darkest pelage, and in general the lighter the coat color, the lighter the eyes. In common with other color phases which have a reduced amount of pigment in the eyes, green-eyed pastels have difficulty in seeing when exposed to bright light, and blink their eyes continuously as if in pain.

Breeders of green-eyed pastels have not reported a tendency toward the nervous reactions called "screw-neck" that is associated with the brown-eyed pastel (*bb*) color phase. A random sample of 48 green-eyed pastels, both male and female, were checked for symptoms of this condition on the Jones ranch in October, 1946; all appeared as well coordinated in their movements as natural dark mink.

TABLE V

BREEDING RESULTS OF THE GREEN-EYED PASTEL COLOR PHASE *

Type Mating	Offspring		Number litters	Average litter size
	Green-Eyed Pastel	Dark		
Green-Eyed Pastel × Green-Eyed Pastel	42	0	14	3.0
Green-Eyed Pastel × Dark (Bgbg)	88	102	48	3.9
Expected (1:1)	95	95		

* Obtained from Mr. Wayne Jones, Ontario, Wisconsin.

The gene (*bg*) for green-eyed pastel behaves as a simple recessive to its non-mutant allele. Data copied from the 1946 breeding records on the Jones ranch are shown in Table V: green-eyed pastel kits only were produced from green-eyed (*bgbg*) × green-eyed (*bgbg*) matings; 48 back cross matings of dark (*Bgbg*) × green-eyed (*bgbg*) gave 88 green-eyed pastels to 102 dark kits, which does not differ significantly from a 1:1 ratio ($X^2 = 1.04$, $P > .30$).

Several breeders have made green-eyed pastel × brown-eyed pastel matings, the resulting kits always being natural dark as would be expected if these color phases result from mutations at different loci. Five individuals which differ phenotypically from either the brown-eyed pastel (*bb*) or the green-eyed pastel (*bgbg*) have been produced on two ranches from *BbBgbg* × *BbBgbg* matings. These pastels are considerably lighter in color than either of the two types of pastel grandparents, and all have red eyes. Although only a small number of these matings have been made, red-eyed pastels which are presumably of the *bbbgbg* genotype have occurred in approximately the proportion expected if *b* and *bg* are on different chromosomes.

Goofus

A pair of dark (wild type) mink produced three very light females in a litter along with dark kits on a small ranch near Winona, Minnesota, " about seven or eight years ago." These three mink appear to be the first representatives of an additional color phase, characterized by a complete lack of pigment in the underfur and a reduction of pigmented guard hairs to approximately one-third the number in the dark mink. The number of pigmented guard hairs varies considerably in different individuals, but the darkest areas are invariably the face, legs and tail in contradistinction to the lighter " points " of the black cross (*Ss*). The lighter individuals of this color phase resemble the Siamese cat.

TABLE VI *

BREEDING RESULTS OF THE GOOFUS COLOR PHASE

Type Mating	Offspring		Number litters	Average litter size
	Goofus	Dark		
Goofus × Goofus	18	0	3	6 0
Goofus × Dark (*Oo*)	11	7	5	3.6
Expected (1:1)	9	9		

* Obtained from Dr. S. S. Osborn, Waterville, Minnesota.

Dr. S. S. Osborn, who purchased a number of these mink in 1946, has termed this color phase " goofus " for want of a better name. The 1947 breeding results on the Osborn ranch are shown in Table VI. Three goofus × goofus matings produced 18 kits, all goofus. Five matings of goofus to darks having one goofus parent gave 11 goofus to 7 dark, which approximates the 1:1 ratio ($X^2 = .88$, $P = .35$). These limited data suggest that the goofus pattern results from homozygosity for the gene *o*.

SUMMARY

Various workers have reported six mutant genes (*p, b, S, F, ip* and c^a) affecting coat color in the ranch-bred mink. In this paper, symbols have been proposed for six additional genes, and the main effects of these in altering the coat color of the dark (wild type) mink to the royal silver ($s^R s$ or $s^R s^R$), colmira (*Cmcm*), ebony (*Ebeb*), aleutian (*alal*), green-eyed pastel (*bgbg*) and goofus (*oo*) color phases have been discussed. Several color phases resulting from combinations of some of these mutant genes have been described. *S* and s^R are alleles; *Eb* and *b* are linked, being approximately 26 cross over units apart.

The twelve mutant genes can be divided roughly into two groups on the basis of their major effects in changing the wild type (dark) coat color to the different color phases. *F*, *S*, s^R, *Cm* and *Eb* when singly substituted for their non-mutant alleles alter the natural dark pelage of the mink by producing a pattern. These patterns for the most part result from white spotting (royal silver, black cross), a differential between the pigmentation of the underfur and the guard hair (ebony), or a combination of both (blufrost, colmira). On the other hand, the genes *p*, *ip*, *al*, *b*, *bg*, and c^H, which produce the recessive color phases platinum, imperial platinum, aleutian, brown-eyed pastel, green-eyed pastel and albino respectively when substituted for their non-mutant alleles, alter the natural dark color by affecting the pigmentation over the entire pelage. The gene for the goofus color phase is the only exception to this rule so far, in that it is recessive to its non-mutant allele, but alters the wild type color by producing a pattern as do the dominant mutant genes.

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LITERATURE CITED

Castle, W. E. and Larry Moore
1946. *Jour. Hered.*, 37: 137-145.
Castle, W. E.
1946. *Jour. Hered.*, 37: 215.
Langenfeld, E.
1947. *Am. Fur Breeder,* 19 (9): 8, 10, 12, 14.
Osborn, S. S.
1946. *Am. Fur Breeder,* 18 (9): 30-32.
Shackelford, R. M.
1941. *Trans. Wis. Acad. Sci. Arts,* Lett., 34: 45.
Shackelford, R. M.
1948. *Genetics,* 33 (3): 161-195.
Shackelford, R. M., and L. J. Cole
1947. *Jour. Hered.*, 38: 203-209.
Smith, S. E., C. H. Whitaker, Leo F. Davids, and Percy V. Noble
1941. *Jour. Hered.*, 32: 173-176.

THE MODIFICATION OF THE EXPRESSION OF A POSITION EFFECT[1]

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Introduction

Many of the phenotypes in *Drosophila* which are due to chromosomal rearrangements have variable expression, being more or less extreme under different conditions (Demerec and Slizynska, 1937; Kaufmann, 1942). Inversion (2LR)40d is of this type (Hinton and Atwood, 1941). The presence of this inversion in *Drosophila melanogaster* can cause an extreme roughening of the facets of the eye, a mottling of the pigment, and the deposition of black tumor-like material on the surface of the eye. However, there is a great variability of expression, ranging from almost wild-type to an effect so extreme that most of the eye is covered over by black material. A study has been made of the factors involved in the production of this variability in the hope that further understanding may be gained of the phenomenon of position effect and its causes.

Other workers (see below) have described several factors that influence the expression of a position effect, yet there is a paucity of exact quantitative correlations between the expression and the influencing factors. The purpose of the present study has been to secure such a quantitative correlation.

In the study of the mottled white eye, Demerec and Slizynska (1937) were able to demonstrate by selection that genetic factors influenced the expression of the translocation which produced the eye condition. Also, the expression was affected by an extra Y chromosome, by the sex of the individual, and by temperature.

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[1] A part of this work was carried out at the Marine Biological Laboratory, Woods Hole. A portion of the study was made with the aid of a grant from the American Cancer Society upon the recommendation of the Committee on Growth of the National Research Council.

Kaufman (1942) found approximately the same to be true of the roughest inversion. The effect of the Y chromosome was earlier shown in other position effects by Gowen and Gay (1934) and Schultz (1936).

The factors tested in the present set of experiments are sex, temperature, age of individual, age of parent, time of hatching, amount of food, Y chromosome, genetic factors, other chromosomal rearrangements, type of food, and rearrangement of the initial inversion. The last two factors will not be discussed in the present paper but are treated separately elsewhere (Hinton, 1948a, b, and c). Preliminary reports of the effect of some of these factors have been presented (Hinton, 1947 and 1948d).

MATERIAL AND METHODS

All studies were made using *Drosophila melanogaster* heterozygous for Inversion (2LR)40d (Hinton and Atwood, 1941). This inversion is lethal in the homozygous condition. Cytologically one break of the inversion is in the left arm of the second chromosome at 26D (Bridges' map), as seen in the salivary gland chromosomes. The other break of the inversion is in the right arm of the second chromosome at 41A. The break in the left arm is in an euchromatic region; the one in the right arm, in heterochromatin.

The variability of phenotypic expression of the inversion was arbitrarily classified into nine categories as follows:

none (o) = no effect. The eye remains completely wild type in facet arrangement and pigmentation.
barely (b) = a very slight roughening of a few facets. The eye is wild type except for a few displaced facets in the lower edge. The pigmentation is normal.
barely + (b +) = a noticeable roughening of the facets but restricted to several small areas. The eye is otherwise normal.
moderate (m) = the roughening of the facets extends over most of the eye but the displacement of the facets is not

extreme thereby giving the appearance of a moderately rough eye. The pigmentation is normal.

very (v) = the facets are markedly displaced and the pigmentation is mottled, producing some areas lighter or darker than others—there may be a few small black deposits on the surface.

¼ = an extremely rough eye with black deposits, mostly in the ventral-most quarter of the eye.

½ = an extremely rough eye with black deposits extending over the ventral-most half of the eye, with the pigment mottled, as above (v).

¾ = an extremely rough eye with black deposits extending over about the ventral-most three quarters of the eye.

full (f) = the entire surface of the eye is covered with the black deposits which may protrude as irregular mounds or growths from the surface.

All classifications were made by the author in order to lessen the subjectivity of such a scheme of classification as much as possible. Only one eye from each fly was classified; the two eyes are usually affected to the same degree.

Chi-square tests were applied to all comparisons of sets of data. Chi-square values up to 9 (P = .02) are usually not considered in this work as indicating a significant difference. Chi-square values above 12.0 (P = less than .01) are considered as indicating a significant difference. However, in all cases, the general shape of the curve of the plotted data seems to be a more reliable indication of existing differences. This seems to be true because in all cases when two curves are compared, they either lie superimposed upon each other (with minor variations) or overlap only at the extremes. There are few cases where an intermediate condition exists.

EXPERIMENTAL DATA

Repeated results. It was found that there was little variability between any two sets of data collected under identical environmental conditions. Fig. 1 shows two populations raised at different times but under identical conditions (in half-pint bottles at 23° C.; 8 pairs of parents of the same age were allowed to lay eggs for three days). The inversion (2LR)40d was balanced with the Curly inversion (In(2LR)Cy) (Bridges and Brehme, 1944) so all offspring carried both inversions. In the population represented by the solid line, 303 eyes were classified. In the population represented by the broken line, 110 eyes were classified. A Chi-square test gives a Probability of about .65 that two such samples would vary as much as this, or more, at random. Thus, it can be shown that the variability in expression of this phenotype falls within certain limits and that the pattern of variability is maintained under similar environmental conditions.

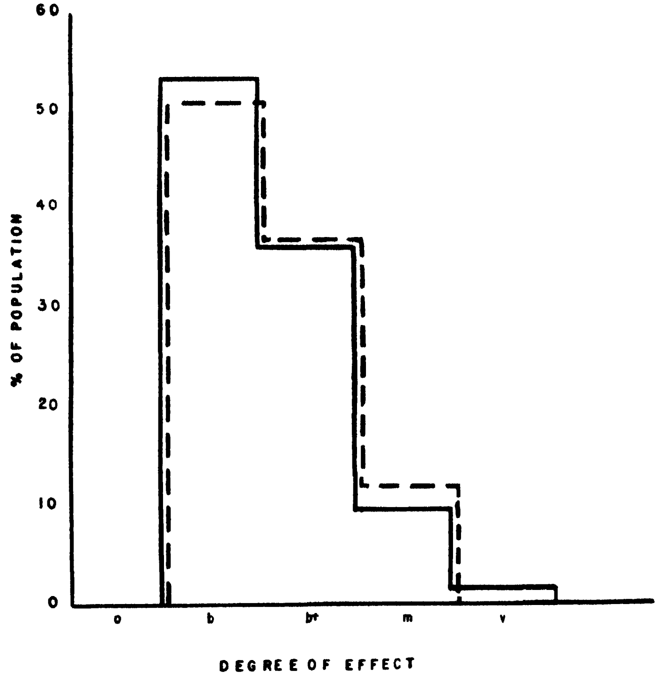

FIG. 1. Two populations raised under identical conditions.

Though the pattern of variability remains the same under similar environmental conditions, that it changes markedly under dissimilar conditions is shown by data which follow. The next step was to define the factors responsible for the change and to attempt to separate them and observe the effect of each independently, in as quantitative terms as possible.

Influence of sex. To see if any differences in effect dependent upon the sex of the individual existed, males and females were classified separately. Fig. 2 shows a population which was separated according to sex (104 males, represented by the broken line, and 132 females, represented by the solid line). A Chi-square test gives a Probability of .05 to .10 that the two sets of data represent the same distribution (Chi-square = 4.97). It appears from this that the sex of the individual has no effect on the expression of this phenotype.

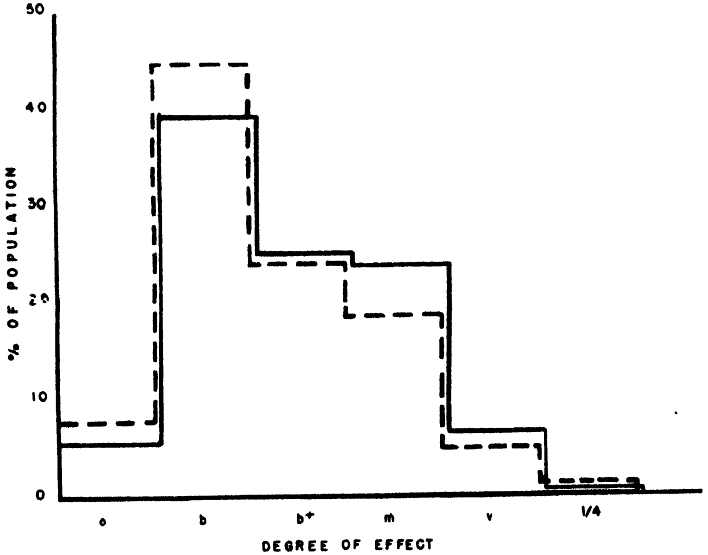

FIG. 2. A population separated according to sex. — — — — — — = Males. —————— = Females.

Age of individual. No change in the degree of expression of the phenotype was found in individuals during their adult life. Young individuals were isolated and the degree of effect on the eye noted. When they were examined two weeks later, the degree of effect remained unchanged.

Age of parents. Crosses were made using young or old males mated to young or old females in all four possible combinations. The same number of parents (8 pairs) was used in each cross. They were given the same amount of culture medium and yeast in half-pint bottles, and all were kept at the same temperature (23° C.); the parents were removed after five days. The offspring were classified and are compared in Fig. 3. From 133 to 222 eyes were classified in each set. Chi-square tests, comparing the sets of data two by two, give Chi-square values of 1.22, 2.90, 5.16, 5.22, 8.84, and 10.10 (P values range from .65 to about .02). Therefore, it appears that there is no significant difference in the phenotype of the offspring whether the parents are young or old.

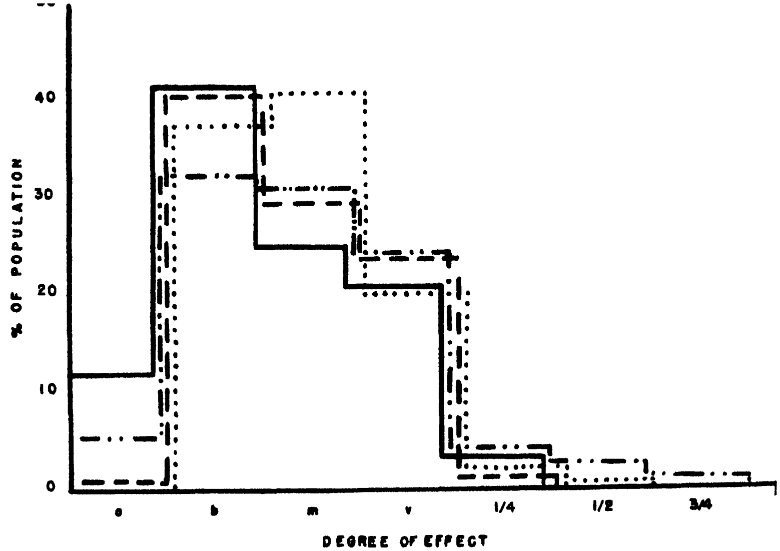

FIG. 3. — — — — — — = Old females × old males. — · · — · · = Young females × young males. · · · · · · · · · · = Old females × young males. ———————— = Young females × old males.

Time of hatching. However, a difference in effect is noted when the offspring which emerge from the pupa cases during the first four days are classified separately from those that emerge during the subsequent week. In Fig. 4 four sets of data (taken from Fig. 3) are grouped together and then divided into two sets according to date of eclosion of the individuals. It can be noted that those offspring which were the last to emerge show a more extreme effect of the eye than do their siblings which hatched earlier. Applying individual Chi-square tests to each of the four sets of data results in Probabilities of less than .01 in three cases and about .03 in the fourth set. [Chi-square values are 12.72 (122 eyes compared to 100), 22.36 (63 compared to 103), 25.55 (67 compared to 56), and 6.30 (63 compared to 123).] These may be considered to be significant differences.

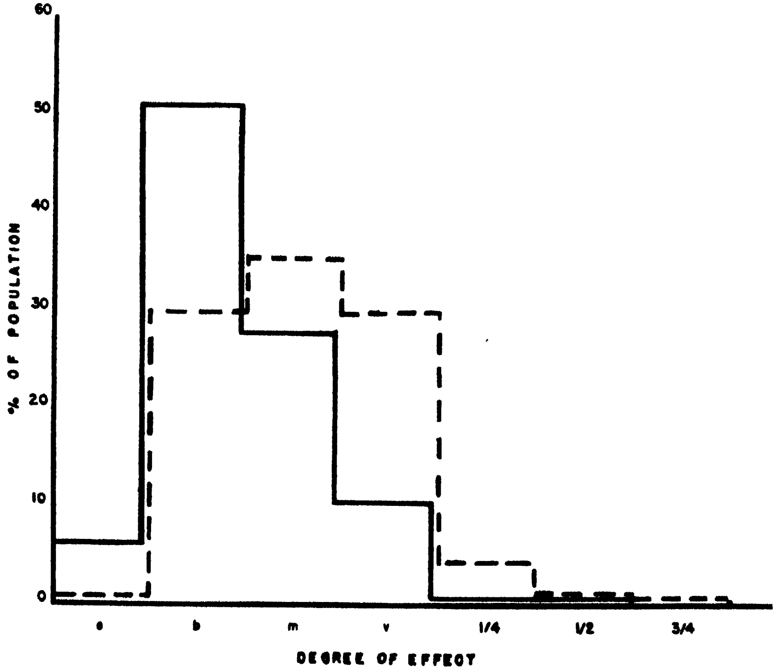

FIG. 4. — — — — — — = Last offspring to emerge from the pupa case. ———————— = First offspring to emerge.

As a further check on this point, additional data were collected from an experiment more precisely organized. Half-pint milk bottles were used with 6 pairs of parents to a bottle. The parents were allowed to lay eggs for one week and were then transferred to a second set of bottles and allowed to lay for one week. The time was noted when the offspring started hatching from the pupa case in each set. At the end of one week after the beginning of hatching, the offspring were classified and discarded. Offspring which hatched later than one week after the first hatch, were classified at the end of two weeks. In this way, four sets of data were obtained: the first hatch in the first set of bottles; the later hatch in the first set; the first hatch in the second set of bottles; and the later hatch in the second set.

Such a set-up should enable one to distinguish between the possibility that the increased effect upon the eye in late hatches was due to a depletion of the food supply, and the possibility that the last eggs a female lays are deficient for some factor necessary to the normal eye. The data are presented in Fig. 5. Chi-square tests comparing the four sets of data give the following results: the first counts on each set compared (467 eyes, and 332) give a Probability of less than .01 ($\chi^2 = 12.7$); the second counts on each set compared (561 eyes, and 584) give a Probability of less than .01 ($\chi^2 = 37.05$); the first count compared to the second count in the first set (467 eyes, and 561) gives a Probability of less than .01 ($\chi^2 = 125.05$); the first count compared to the second count in the second set gives a Probability of less than .01 ($\chi^2 = 153.5$); the first count of the second set compared to the last count of the first set gives a Probability of less than .01 ($\chi^2 = 69.8$); and the first count of the first set compared to the last count of the second set gives a still lower Probability ($\chi^2 = 248.8$). It can safely be concluded that there is a significant difference between the first and second counts in each set. Also there is a significant difference between the two first counts, and between the two second counts. Therefore, both the food and the eggs have an influence on the eye.

Crowding. Two types of containers were used in this experiment for raising the flies. One was a five-inch shell vial and the other a half-pint sized bottle. Eight pairs of parents were placed in each type of container. The cultures were raised at 28°. From the vial, 124 offspring were ob-

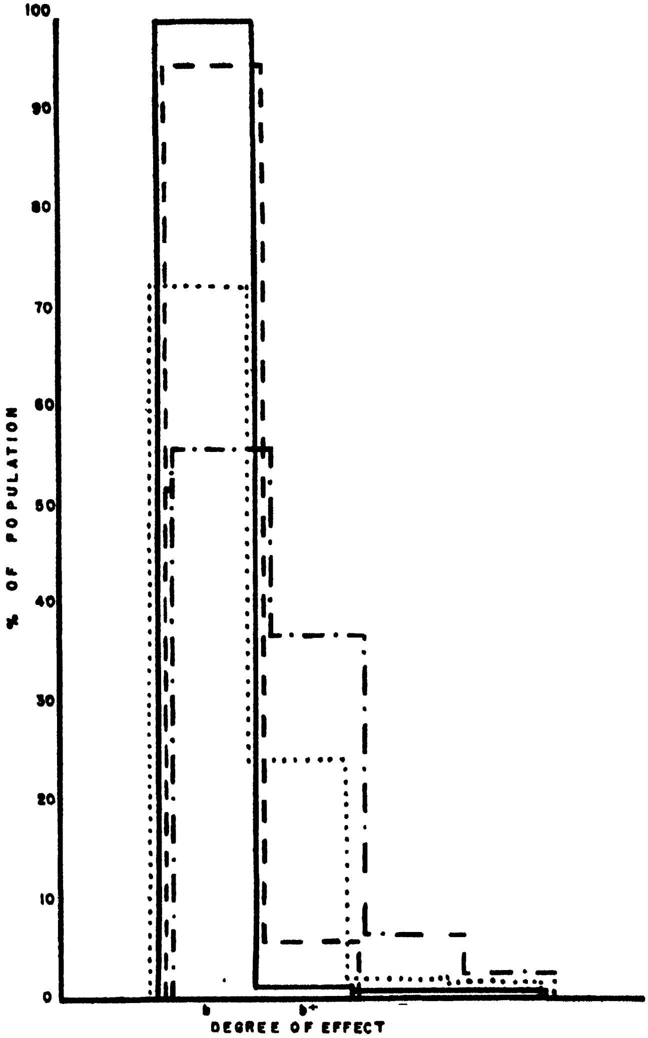

FIG. 5. ———————— = First offspring in first bottles. — — — — — = First offspring in second bottles. · · · · · · · · · · = Second offspring in first bottles. · — · — · — · = Second offspring in second bottles.

tained and the eyes classified; from the bottle, 458 offspring. A striking difference in the degree of eye effect in the two sets can be noted by comparing the two distribution curves (Fig. 6). A Chi-square test shows that there is a Probability of less than .01 that these are related sets of data.

The flies raised in the shell vial show much more extreme effect than those raised in the bottle under less crowded conditions.

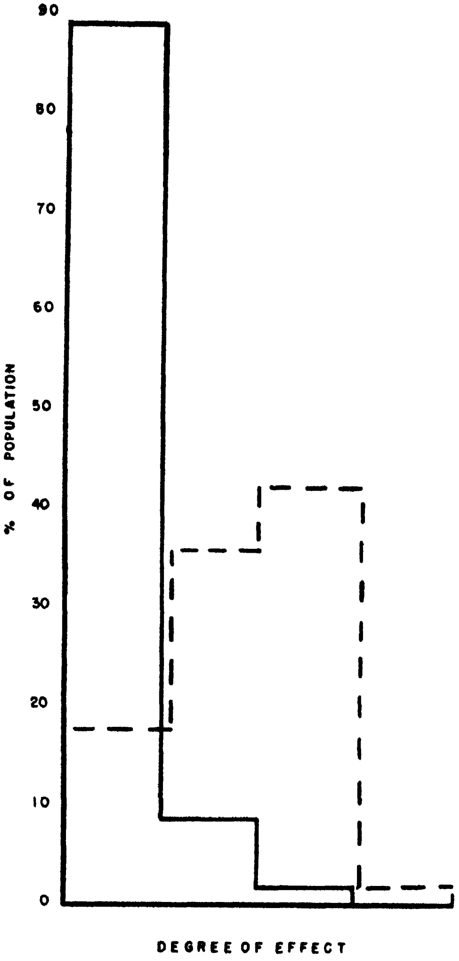

FIG. 6. ——————— = Flies raised in half-pint sized bottles. — — — — — — = Flies raised in 5-inch shell vials.

Temperature. Culture bottles (half-pint) containing 8 pairs of parents were kept at each of the following temperatures during the entire development of the offspring: 15°, 17°, 19°, 23°, 25°, and 28°. The results of the effect of the various temperatures on the eyes of the offspring are shown by the distribution curves in Fig. 7. At 15°, there was relatively little expression of the position effect. A statistically significant increase in effect is shown when the temperature is raised by two degrees ($P =$ less than .01, $\chi^2 = 28.25$—186 flies compared to 164). A still greater effect is gained at 19° ($P =$ less than .01, $\chi^2 = 20.41$—186 compared to 101). However, when the temperature is raised to 23°, the curve resembles that obtained at 17° ($P =$ about .20, $\chi^2 = 4.84$—164 compared to 110). At 25° the situation obtained at 15° is somewhat approached ($P =$ about .01, $\chi^2 = 18.45$—186 compared to 272); while at 28°, the picture is almost identical with that obtained at 15° ($P =$ about .75, $\chi^2 = 1.28$—186 compared to 137). It appears that the eye effect is lessened at both extremes of temperature, the expression being most marked at the intermediate temperatures.

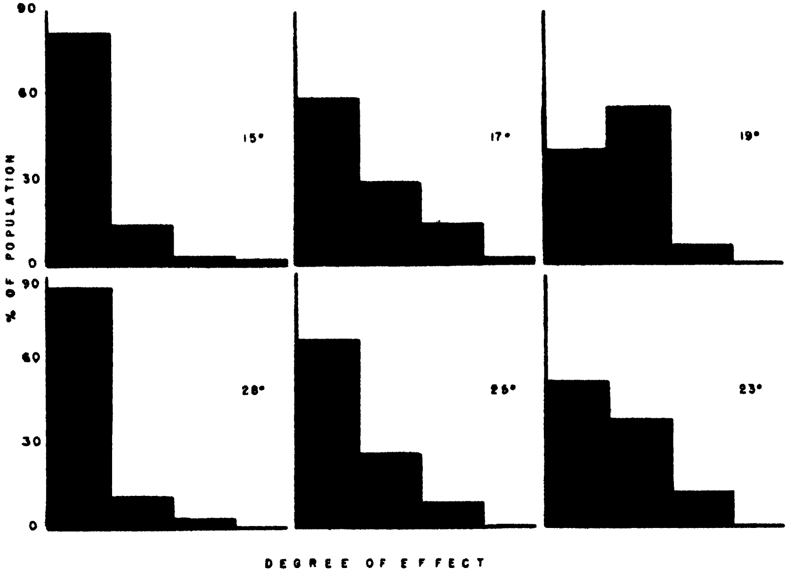

FIG. 7. Populations raised at various temperatures.

Extra Y-chromosome. Flies carrying Inversion (2LR)40d were mated to females known to have "attached" X-chromosomes and a Y-chromosome (±/w). The female offspring (aside from the triple-X "superfemales") would necessarily receive, in addition to the normal number of chromosomes, a Y-chromosome from the male parent. All the male offspring would receive the normal number of chromosomes. A comparison is made in Fig. 8 of the normal male offspring to their sisters carrying the extra Y-chromosome. These data show a significant difference ($P =$ less than .01, $\chi^2 = 49.6$—63 flies compared to 66) in the expression of the inversion. The expression is much less extreme in the case of the females with the Y-chromosome.

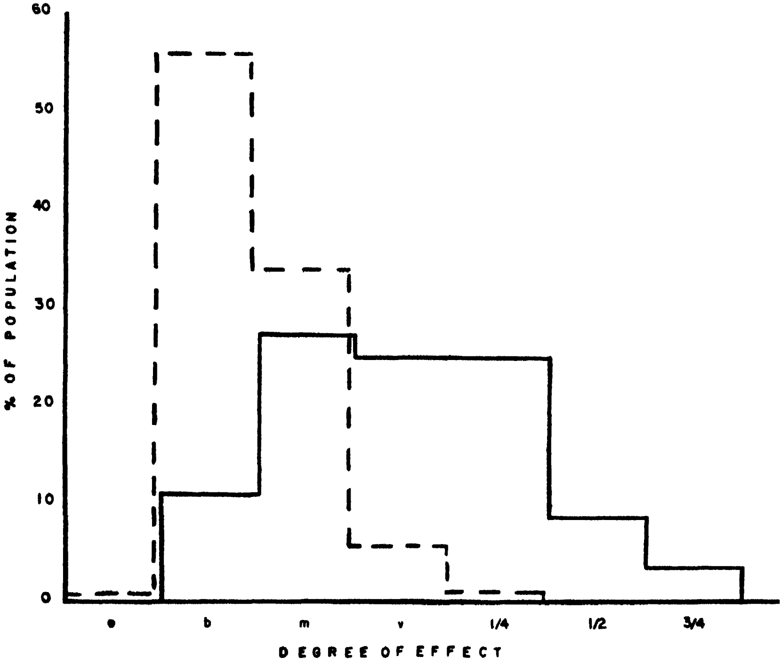

FIG. 8. ———————— = Normal male offspring. — — — — — — = Sisters with extra Y-chromosomes.

Genetic modifiers. It was noted that in some cases when In(2LR)40d was crossed to another stock of *D. melanogaster*, the resulting offspring showed a more extreme effect from the inversion than the parents had shown. This was

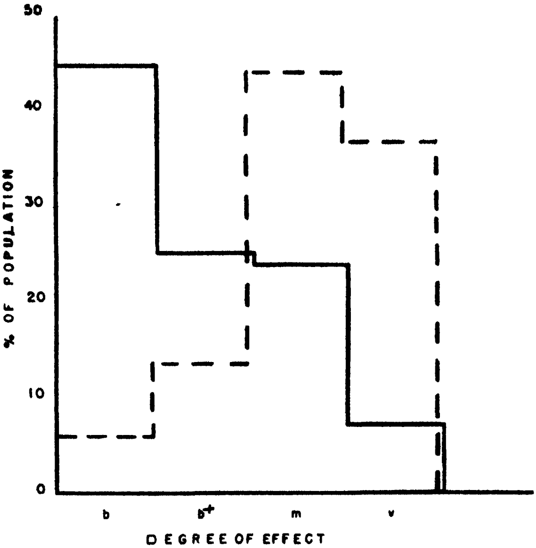

FIG. 9. ———— = Offspring of In(2LR)40d inbred. - - - - - = Offspring of In(2LR)40d × Cy/Pm ; H/SbC.

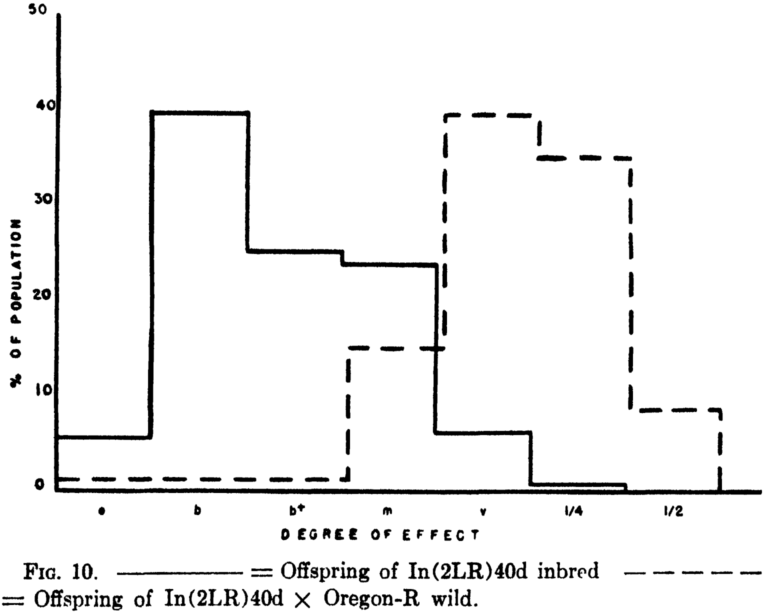

FIG. 10. ———— = Offspring of In(2LR)40d inbred - - - - - = Offspring of In(2LR)40d × Oregon-R wild.

especially true in the cases of crosses with Cy/Pm; H/SbC (Fig. 9) and with Oregon-R wild (Fig. 10). The probabilities of their being samples from the same population are less than .01 in both cases (Fig. 9: $\chi^2 = 31.3$—236 flies compared to 68; Fig. 10: $\chi^2 = 198.7$—236 compared to 98).

However, this is not always the case, since in some outcrosses the phenotype of the offspring remains the same as that of the In (2LR)40d parents regardless of the introduction of genetically different chromosomes. Such is the case when In(2LR)40d is crossed to Bl L^4/Cy (Fig. 11). The Probability that they are the same is about .15 ($\chi^2 = 5.86$—236 flies compared to 111).

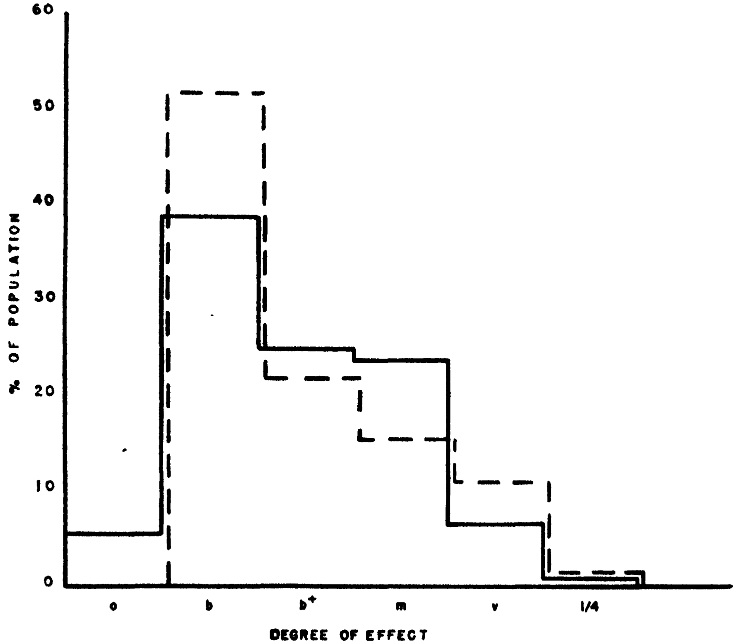

Fig. 11. ———— = Offspring of In(2LR)40d inbred. — — — — — = Offspring of In(2LR)40d × Bl L^4/Cy.

An attempt was made to identify the factors responsible for the change in phenotypic expression which occurred in the offspring of the above-mentioned crosses. For this purpose two series of crosses were carried through. The first of these was a cross intended to produce offspring which would carry all the chromosomes of the In(2LR)40d parent except for the Curly chromosome. This cross is outlined as follows: the wild type chromosomes of the In(2LR)40d parent are represented by an encircled plus sign for convenience in distinguishing them from the wild type chromosomes of other stocks. Each individual is represented by three pairs of chromosomes, the first, second and third, written from left to right in that order. (The small fourth chromosomes are ignored in these crosses.)

TABLE 1

+ ; Cy/Pm ; H/SbC × In(1)dl49/sc ec ; +/+ ; +/+
⊕/⊕ ; In(2LR)40d/Cy ; ⊕/⊕ × In(1)dl49 ; Cy/+ ; SbC/+
In(1)dl49/⊕ ; In(2LR)40d/+ ; SbC/⊕ × ⊕ ; In(2LR)40d/+ ; SbC/⊕
⊕/⊕ ; In(2LR)40d/+ ; ⊕/⊕

The phenotype of the final offspring (G) (those identical

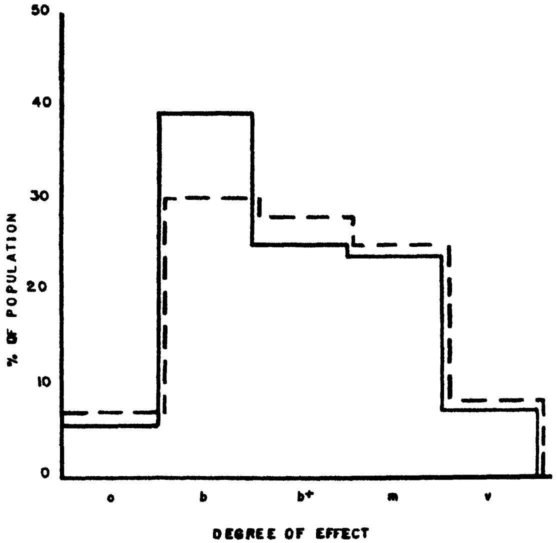

Fig. 12. ———— = ⊕/⊕ ; In2LR/(Cy) ; ⊕/⊕. — — — — — = ⊕/⊕ ; In2LR/+ ; ⊕/⊕.

with the In(2LR)40d parents except that they lack the Curly chromosome) were compared to the In(2LR)40d parents (A) shown in Fig. 12. No statistically significant difference exists as indicated by a Probability of .85 ($\chi^2 = .95$—236 offspring in A; 43 offspring in G).

The significance of these results will be dealt with in the discussion.

The other series of crosses is outlined as follows:

TABLE 2

⊕ ; In(2LR)40d/Ⓒy ; ⊕/⊕ × +/+ ; Cy/Pm ; H/SbC
⊕/+ ; In(2LR)40d/Cy ; ⊕/SbC × + ; Ⓒy/Pm ; ⊕/SbC
⊕ or +/+ ; In(2LR)40d/Ⓒy ; ⊕/⊕

The final offspring (F) of this series of crosses were compared to the In(2LR)40d stock (A) as shown in Fig. 13. There is probably no significant difference in the phenotypic expression in the two cases (P = about .20) ($\chi^2 = 4.76$—23 offspring of type F were classified).

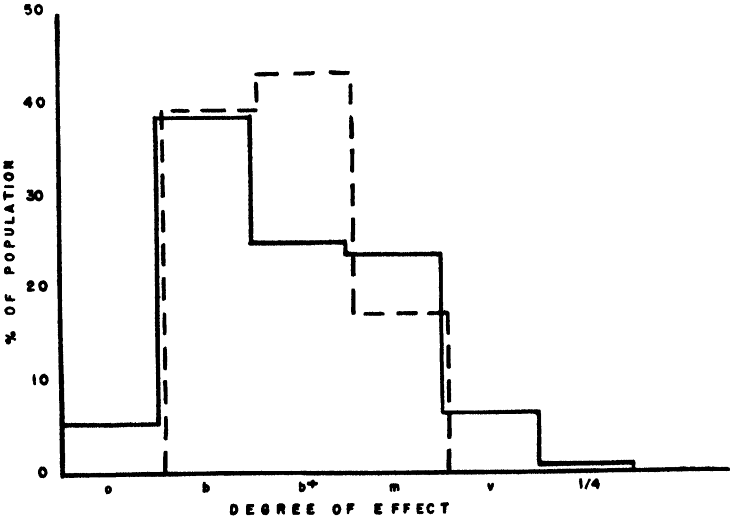

Fig. 13. ——— = ⊕/⊕ ; In2LR/Ⓒy ; ⊕/⊕. – – – – – – = ⊕/+ ; In2LR/Ⓒy ; ⊕/⊕.

DISCUSSION

In this series of experiments we are dealing with a chromosomal inversion which has a phenotypic expression upon the eye, a position effect. The degree of expression varies from no effect to a very extreme effect depending upon a number of factors. The series of experiments reported here was designed to test the influence of some of these factors upon the degree of expression of the inversion.

From the results, several possible factors can, at the onset, be ruled out; these having little or no influence upon the position effect. These factors are sex, age of the individual, and age of the parents.

The phenotype produced by the inversion is significantly more extreme, however, in crowded cultures. This may be a starvation effect, although the present evidence does not rule out other explanations.

Also to be noted is that the last individuals to emerge from the pupa cases show a more extreme phenotype than do their siblings. This is not due to the age of the parents as has already been shown. It is possible that the last eggs a female lays are deficient for some substance which is necessary for its phenotype to approach the normal. It is also possible that the food source is not as abundant for the later larvae and the extreme phenotype is actually due to a starvation effect just as it was in the case of crowded cultures. Both of these possibilities seem most likely in light of the experiment the results of which are summarized in Fig. 5. In this experiment two factors were noted: the age of the culture medium at the time of hatching, and whether the eggs were among the first or last to be laid by the parents. It appears from the results that the latter of these factors plays a lesser but noticeable role.

For convenience in discussing, the four sets of data are numbered, as follows: (1) first count in first set of bottles; (2) last count in first set of bottles; (3) first count in second set of bottles; (4) last count in second set of bottles.

If the depletion of some ingredient of the food were the sole factor influencing the increased effect on the eye, it would be expected that counts 1 and 3 would give a similar distribution and that counts 2 and 4 would be similar to each other. If a progressive deficiency in the last eggs of some necessary substance were the sole factor, it would be expected that the effect on the eye would be progressively more severe from count 1 to 4 in the ascending order of 1, 2, 3, 4. However, if both of the above discussed factors were having an influence, it would be expected that the severity of effect would ascend in the order of 1, 3, 2, 4. The Chi-square tests should show a difference between each two sets with less difference between 1 and 3 and an increasing difference in the following order:

1 – 3
2 – 4
3 – 2
1 – 2
3 – 4
1 – 4

When the Chi-square values are arranged in that order, the results are as follows:

12.7
37.05
69.8
125.05
153.5
248.8

The agreement of prediction with results strongly indicates that both factors are influencing the results, namely, that some substance necessary to the normal eye becomes depleted in the food and also in the last eggs laid by the female.

The possibility still remains that some effect is due to a slower development. The influence of the rate of development on mosaics has been indicated by Noujdin (1946). An attempt was made to examine this possibility in the present investigation. Fifty eggs of the same age were placed individually in 50 shell vials with ample medium.

The date of hatching from the pupa case was noted and the eyes examined to see if the last to hatch were more severely affected by the inversion than were the first to hatch. Only 14 of these pupated, and all on the 7th day. They hatched over a period of three days. However, only 3 hatched on the first day and only one on the 3rd, the remaining hatching on the second day. There was not enough difference in the effect on the eye to justify any conclusion; all flies were either barely affected or barely +.

It, therefore, still remains a likely explanation that the influencing factor is the availability of some essential substance. The exact nature of this situation is being tested by raising cultures carrying In(2LR)40d under aseptic conditions on chemically defined medium. The results of these tests will be reported at a later date.

The analysis of the genetic modifiers involved is far from complete, yet certain points appear to be well established. The presence of genetic modifiers seems to be the only interpretation that can be placed upon the results obtained from the crosses, the offspring of which are shown in Figs. 9, 11 and 12.

In the second of these crosses offspring (B) were obtained which differed from the parents (A) in only one respect. That is, one of the wild type X and 3rd chromosomes, and the Curly second chromosome were replaced by chromosomes from the Bl L^4/Cy stock. This apparently had no effect upon the expression of the inversion since these heterozygous offspring show no statistically significant different distribution from the parents. However, when the same parental stock (A) was crossed to Oregon-R wild (D), the offspring (Fig. 10) showed a striking increase in effect. These offspring, like the ones in Fig. 11, had had one wild type and 3rd chromosome replaced by a wild type X and 3rd from another stock. However, they differed in that in these offspring the Curly inversion had been replaced by a wild type second. These two sets of offspring have an identical chromosomal picture except for the lack of the

Curly inversion in the latter set. The only other difference lies in the different origin of the wild type chromosomes involved in the two sets.

The question, therefore, arose as to whether the difference in phenotypic expression was due to the absence of the Cy inversion, or to the genetic constitution of the wild type chromosomes. To help answer this question, a series of crosses was made (as described in Table 1) to obtain offspring (G in Fig. 12) which had all chromosomes from the parental stock (A) with the exception of the Curly inversion. If presence or absence of the Curly inversion is responsible for the change in expression, these offspring should show an extreme effect in Fig. 10. However, as Fig. 12 shows, there is no significant increase in effect and the conclusion is drawn that it is not the absence of the Curly inversion which has an effect but rather the genetic nature of the wild chromosomes. Apparently the Bl L^4/Cy stock carries different modifiers than does the Oregon-R stock, and these modifiers in the two stocks have a different effect on the expression of Inversion (2LR)40d.

There is some indirect evidence to indicate that the modifiers, at least in one case, may be on the third chromosome. It can be noted in Fig. 9 that offspring (C) obtained from a cross of the parental stock (A) to Cy/Pm; H/SbC show a significant increase in effect, even though the second chromosomes remain identical to those in the parental stock.

A series of crosses (as outlined in Table 2) were made to obtain offspring (F in Fig. 13) with both the second and third pairs of chromosomes identical with the parental stock (A) and only the X-chromosomes being derived from the Cy/Pm; H/SbC stock. When offspring of that type are compared to the parental stock (Fig. 14) no significant increase is noted. Therefore, it can be concluded that the X-chromosome does not carry modifiers which are capable (at least alone) of enhancing the expression of Inversion (2LR)40d. The effective modifiers, therefore, by process of elimination, would be supposed to be on the third chromosome. There is no positive evidence that they are on the third chromosome, and there is no evidence to indicate whether the modifier is a single gene or a series of genes.

At both 28° C. and 15° C. the phenotype is close to normal, but is more extreme at intermediate temperatures. There appears to be some optimum temperature at which the inversion can express itself to the extreme. A variation in temperature in either direction from this point causes the effect to be less extreme.

It is always a temptation to search for some basic factor that all the effects have in common. Such a factor is difficult·to find in these experiments which show that food supply, temperature, and genetic environment all effect the phenotypic expression of the inversion. However, if the exact nature of the inversion is realized, it at least offers a clue. It should be remembered that the inversion (2LR)40d has one break in heterochromatin and the other in the euchromatic region 26D/E. The resulting configuration is one in which the euchromatic region 26D (distal to the break) and the euchromatic region 26E (proximal to the break) each lie next to heterochromatin. It has been shown (Hinton, 1948a, b and c) that this inversion can produce its characteristic phenotype only when the heterochromatin is in this position. Even rearrangements of the heterochromatin to distances close to, but not adjacent to, region 26D/E cause the phenotype to be normal. Therefore, the position of the heterochromatin appears to be the primary factor involved in this position-effect. If this is true then we might expect any environmental factor which alters the expression of the inversion to alter it by first affecting the heterochromatin.

" U-shaped " curves, like the temperature curve, are not unknown in situations involving heterochromatin. Prokofyeva-Belgovskaya (1945) obtained a similar curve when the amount of heterochromatization in the X-chromosome was studied at temperatures ranging from 14° C. to 30° C.

This recalls the curve, found by Plough (1917) (corrected by Smith, 1936) showing that crossing over is appreciably increased at the two ends of the temperature range. Since it is known that extra Y-chromosomes also increase crossing over (Stern, 1936), there is probably a correlation between heterochromatin and crossing over, and it is possible that the rise in crossing over is due to a temperature effect upon the same heterochromatin mechanism operating in the other cases. This would seem to indicate that heterochromatin is at its maximum efficiency at extreme temperatures if more frequent crossing over and more normal phenotypes may be considered as criteria of a more efficient system. However, it is at the extremes of temperature that Prokofyeva-Belgovskaya finds the least heterochromatization. Therefore, it seems more logical to form an hypothesis based on an inhibitory effect of heterochromatin. It may be assumed that some of the heterochromatin prevents a process basic to such phenomena as crossing over, and that if part of this type of heterochromatin is inserted next to certain normal genes, it may also inhibit their expression (producing a position effect). If this inhibitory effect is enzymatic in nature it would not be surprising to find that it had an optimum temperature at which it would act, and that at extreme temperatures it would fail to exert its inhibitory effect. That would account for the rise in the frequency of crossing over and the return to the normal phenotype in the present data at the extremes of the temperature range. The fact that extra Y-chromosomes act in the same way as extreme temperatures would seem to indicate that they have the capacity to block the inhibiting action of the other heterochromatin. That the heterochromatin of the Y-chromosome is different from the heterochromatin that we see in salivary gland chromosomes is evidenced by the very fact that the heterochromatin of the Y-chromosome can scarcely be seen at all in salivary gland preparations.

Just as one would not expect all genes to act in the same way, one would not expect translocated heterochromatin to affect all genes similarly; nor extra Y-chromosomes and temperature to affect all position effects in a like manner. This expectation is borne out by the variety of curves found in the literature describing effects on the expression of position effects (Demerec and Slizynska, 1937; and Stern, MacKnight and Kodani, 1946, as examples). This may be interpreted to mean that the activity of a gene is correlated with its proximity to heterochromatin and that the action of a gene is inhibited if its normal relationship to heterochromatin is altered. However, since some genes seem to be hypomorphic and others hypermorphic, the end result of the inhibition would necessarily be different in different cases.

It could well be supposed that the pairing of the chromosomes is one of the processes which is referred to as being inhibited in the foregoing hypothesis. If pairing were partially inhibited at normal temperatures, crossing over might well be limited as a result. An extreme temperature or an extra Y-chromosome would overcome the effect and allow more exact pairing, thereby increasing the possible amount of crossing over. That pairing is influenced by extra Y-chromosomes is indicated by the observation of Cooper (1948) that pairing in the presence of an extra Y-chromosome was much more complete in the giant neuroblast cells of the brain ganglia of *Drosophila* than it was observed to be by Hinton (1946) when, supposedly, the same type of cells were studied without the extra Y-chromosome. Also this idea would be in agreement with the position effect data if it is assumed that the inserted heterochromatin in some way upsets normal gene behavior by upsetting pairing in that region. This idea has previously been introduced by Ephrussi and Sutton (1944), and Gersh and Ephrussi (1946). By this reasoning, pairing would seem to be dependent upon some basic chemical process (probably enzymatic) involving the heterochromatin of the chromosome.

Unfortunately, the nature of heterochromatin is not sufficiently understood to allow an exact interpretation of the mechanism by which the heterochromatin acts. It is known, however, that heterochromatin differs from euchromatin in its nucleic acid metabolism (Schultz, Caspersson and Aquilonius, 1940). It is conceivable that the factors discussed are, in some way, influencing the nucleic acid metabolism. It would be expected that temperature would have an influence on such a process; that extra Y-chromosomes would; also that starvation or the absence of nucleic acid in the diet would enhance the effect. And it is not too inconceivable that genetic factors might also modify the activity by working through such channels.

SUMMARY

A second chromosome inversion (In(2LR)40d) in *Drosophila melanogaster* causes an effect on the eye of the adult fly. This phenotype is variable in its expression under different conditions. An investigation has been made to determine the relative roles of various factors in the variability of expression. It is concluded that:

(1) If two sets of offspring are raised under identical conditions, the distribution of degree of effect will be the same (Fig. 1).
(2) The sex of the offspring has no effect on the degree of expression (Fig. 2)
(3) The degree of effect does not change with the age of the individual.
(4) The age of the parent is not a factor in determining the degree of effect (Fig. 3).
(5) The last offspring to hatch from the pupa case show a more extreme effect than do the first offspring to hatch (Fig 4)
(6) Offspring raised in crowded cultures are more extremely affected than offspring raised in cultures with abundant food (Fig. 6).
(7) Offspring from the last eggs laid by the mother are more extremely affected than those from the first eggs (Fig 5).
(8) Offspring raised at the extremes of the temperature range (15° C. and 28° C.) are less affected than those raised at the intermediate temperatures (Fig. 7).
(9) A Y-chromosome in the female reduces the effect as compared to the normal brothers (Fig. 8).
(10) Genetic modifiers may increase the effect. These are not present in all stocks. In one stock studied, they appear to be on the third chromosome (Figs. 9–13).

It is suggested that all these factors affect the phenotype by working through the heterochromatin and possibly through the nucleic acid metabolism of the region of the chromosome involved in the inversion.

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LITERATURE CITED

Bridges, C. B., and K. S. Brehme
1944. Carn. Inst. Wash. Pub. 552.
Cooper, K. W.
1948. *Jour. Exp. Zool.*, 108: 327–336.
Demerec, M., and H. Slizynska
1937. *Genetics,* 22: 641–649.
Ephrussi, Boris, and Eileen Sutton
1944. *Proc. Nat. Acad. Sci.,* 30: 183–197.
Gersh, E. Sutton, and Boris Ephrussi
1946. *Proc. Nat. Acad. Sci.,* 32: 87–94.
Gowen, J. W., and E. H. Gay
1934. *Genetics,* 19: 189–208.
Hinton, Taylor
1946. *Jour. Exp. Zool.,* 102: 237–252.
1947. *Biol. Bull.,* 93: 216.
1948a. *Genetics,* 33: 108.
1948b. Proc. 8th Int. Cong. Gen. (in press).
1948c. *Rec. Gen. Soc. Am.,* 17: 40.
1948d. *Anat. Rec.,* 101: 21.
Hinton, Taylor, and K. C. Atwood
1941. Carn. Inst. Wash. Year Book, 40: 231–232.
Kaufmann, B. P.
1942. *Genetics,* 27: 537–549.
Noujdin, N. I.
1946. *Doklady Akademia Nauk USSR,* 14: 61–63
Plough, H. H.
1917. *Jour. Exp. Zool.,* 24: 147–209.
Prokofyeva-Belgovskaya, A. A.
1945. *Zhournal Obshchei Biologie,* 6: 93–124.
Schultz, J.
1936. *Proc. Nat. Acad. Sci.,* 22: 27–33.
Schultz, J., T. Caspersson and L. Aquilonius
1940. *Proc. Nat. Acad. Sci.,* 26: 515–523.

Smith, H. F.
1936. *Nature,* 138: 329–330.
Stern, Curt
1936. *Genetics,* 21: 636–730.
Stern, Curt, R. H. MacKnight and M. Kodani
1946. *Genetics,* 31: 598–619.

ON CORRELATION OF THE PHENOMENA ASSOCIATED WITH CHROMOSOMES, FOREIGN PROTEINS AND VIRUSES

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INTRODUCTION

DISCOVERIES of the last decade are casting a new light on the seemingly heterogeneous phenomena associated with chromosomes, foreign proteins, and viruses. Being intrinsically linked with proteins, it is to be anticipated that these phenomena will ultimately be explicated upon the basis of common factors. It has thus seemed desirable to examine them in the light of new information and to attempt to correlate them upon the basis of factors which appear to be basic.

In 1922 Muller suggested that a specific attractive force between homologous chromosomes brings about their pairing during meiosis. These chromosomes may be separated from one another by distances of the order of a micron at the beginning of the pairing process (Fabergé, 1942; Hinton, 1946). For this reason this force is referred to as a *long range* specific attractive force (hereafter referred to as LRSAF), thereby distinguishing it from the short range specific attractive forces which act between molecules at separations of at most a few angströms. In recent years the LRSAF has been the subject of considerable discussion and investigation (*cf.* Bernal, 1940; Bernal and Fankuchen, 1941; Cooper, 1948; Friedrich-Freksa, 1940; Hinton, 1946; Jehle, 1948; Jordan, 1940; Langmuir, 1938; London, 1943; Muller, 1941; Oster, 1947; Pauling and Delbrück, 1940; Vlasow, 1945; Wrinch, 1947). Chromosome behavior provides the most convincing indirect evidence for the existence of such a force. In the last few years Rothen (1947, 1948) has obtained direct experimental evidence that " the interaction between large molecules, such as those involved in immunological and enzymatic reactions, may take place through a field of specific long-range forces extending over 200 Å." Although the evidence for a LRSAF is not conclusive, it seems sufficient to warrant their consideration in any discussion of protein-associated phenomena. In the subsequent discussion the existence of such forces has been assumed as a working hypothesis.

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If a LRSAF exists between greatly similar and identical protein particles, it is clear that factors which counteract intermolecular attraction should be of particular significance in determining its effects. A primary factor in this category is the coulomb repulsion between protein particles in solution. This repulsion results because of the great electrostatic charges carried by proteins and it is minimized at the pI (isoelectric pH) of the particles concerned. For this reason it is to be anticipated that the pH of protoplasm will influence the effects of long-range attraction. A second factor which tends to prevent the association of attracting particles is their brownian or thermal velocity.

In cases where particle association mediates protein synthesis, the availability of energy and materials from ancillary reactions or directly from the protoplasm itself is of great significance. This is becoming increasingly evident from present-day studies which clearly indicate the prime role played by nucleic acid in protein synthesis.

I. CHROMOSOME ACTIVITY

Three fundamental forms of chromosome activity present themselves: synthesis of metabolites, self-duplication, and pairing. The LRSAF is assumed by its proponents to be effective, not only between greatly similar and identical proteins, but also between proteins and their constituent *proteons* or *subproteons*.[1] The numerous experiments of Landsteiner (1946) and his collaborators upon the adsorption of haptens by antibodies have shown directly that very simple molecules can be specifically adsorbed by proteins. The essence of well-known hypotheses (*cf.* Alexander and Bridges, 1928; Haldane, 1935; Koltzoff, 1938; Muller, 1922, 1941; Troland, 1917; Wright, 1941, 1945; Wrinch, 1947) is that genes or subgenes specifically adsorb subproteons and then act as a templet guiding the synthesis of these molecules into replica proteins.

The above action appears to be an essential feature of both metabolite synthesis and gene duplication. While this hypothesis of the synthetic process is acceptable it remains to account for the elaboration of the synthesized metabolites for, as Wright (1941) has remarked, the duplication must be associated with a mechanism of separation. The same question naturally arises with regard to the separation features of chromosome disassociation and disjunction. In this respect Darlington (1937) has suggested that changes in the pH of the protoplasm during mitosis and meiosis alter the magnitude of the coulomb repulsion between the similarly charged homologous chromosomes and the centromeres (kinetochores) and centrosomes. Numerous experiments (*cf.* Schrader, 1944) have shown that the chromosomes behave as if negatively charged, and Botta (1932) is convinced that the magnitude of this charge varies with the different phases of cell division. Indirect evidence of changing surface charges on different cell-organs is provided by the changing fixation-staining reaction and the hydration-dehydration and chromosome spiralization cycles. Cyclic changes in the surface charge in turn imply a cycle of protoplasmic pH changes (Darlington, 1937; Kuwada, 1939). Thus, for example, the dehydration of previously more dispersed chromatin which renders the chromosomes visible at the end of the resting stage (Darlington, 1937) is believed to be associated with a decrease in the nucleoplasmic pH (Sakamura, 1927). Direct evidence of such changes is afforded by the work of Yamaha (1935), according to which the pH of the nucleoplasm and cytoplasm undergoes cyclic changes during meiosis in *Tradescantia reflexa, Lilium speciosum,* and other plant species. Thus, *varying* coulomb repulsion probably accounts in large part for the mechanics of chromosome separation. To account for metabolite elaboration, however, it is necessary to consider other mechanisms, since this process can occur in cells constantly in a certain mitotic phase, presumably at constant pH, as, for example, the constant mitotic prophases of glandular secretory cells.

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[1] We adopt the terminology of Wrinch (1947) wherein the term *proteon* connotes a native protein unit incapable of division into subunits also having the native protein character. A native protein is then regarded as a proteon or a system of proteons. A *subproteon* is a functional subunit of the proteon chain and might itself be a proteon of a different protein. The term *metabolite* is used in reference to the substances whose synthesis is directly mediated by the chromosomes or by nucleoproteins present elsewhere in the cell.

METABOLITE SYNTHESIS AND ELABORATION

The elaboration of metabolite molecules after their synthesis might occur automatically as a direct result of factors brought into play by the synthetic process. Since metabolite synthesis results in a replica of the parent templet, the metabolite and the templet will have essentially identical pI's. Thus, they will carry the same charges at non-isoelectric pH's of the nucleoplasm. Perhaps it is the coulomb repulsion to which these charges give rise which is the cause of metabolite elaboration. This repulsion of the metabolite would not be anticipated in the interaction of the templet with the subproteon building blocks used in the synthesis. The subproteons have different pI's from those of the templets to which they are attracted, so that those which carry lesser charges of the same sign or charges of opposite sign could be adsorbed. Moreover, the subproteons may be adsorbed as derivatives, so that, for example, in the absence of free carboxyl groups, ionization of an amino acid derivative as the anion might not occur and this could obviate coulomb repulsion from the negatively charged chromosomes.

The synthesis and elaboration of metabolites by chromosomes might, then, be visualized as proceeding in the following manner. The LRSAF attracts nucleoplasmic subproteons to the genic templets, where they are adsorbed as specific short-range forces become effective. Under the influence of these templets, and with the required energy available from ancillary reactions or from one of the reactants, the subproteons are synthesized into metabolite molecules. These are immediately ejected from the chromosome as a result of the coulomb repulsion which arises between them and the parent templets.

It is interesting in connection with the above discussion to note the behavior of yeast volutin granules (these are associated with ribose nucleic acid, Brandt, 1941; Caspersson and Brandt, 1940). As the yeast cells enter the growing phase the volutin granules are observed to swell, fragment and gradually disappear in the cytoplasm. This action appears to occur simultaneously with the assumption of synthetic elaborational activity by these granules. It would appear that the volutin granules are aggregates of a large number of identical smaller particles, which repel one another resulting in fragmentation because of the coulomb repulsion which sets in as the cytoplasmic pH leaves the isoelectric zone.[2] After the growing phase of synthesis and elaboration (analogous to metabolite synthesis and elaboration by genes), the pH of the cytoplasm returns to the initial value. The granule particles distributed throughout the cytoplasm, since they now repel one another to a lesser extent, are brought together under the action of the LRSAF, reforming volutin granules. The swelling of the volutin granules before their fragmentation is probably due to their becoming hydrated (hydration-dehydration cycles are known to occur in certain cellular components, *cf.* Kuwada, 1937, 1939). This indicates that the pH of the cytoplasm is changing, for, as Fujii (*cf.* Kuwada, 1939) has pointed out, hydration-dehydration cycles must be intimately associated with a changing pH.

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[2] Claude (1943) has observed that the sedimentation rate of the cytoplasmic "small particles" or microsomes is particularly influenced by the pH of the medium.

CHROMOSOME DUPLICATION AND PAIRING

It seems unlikely that there should be any fundamental difference between two such closely related phenomena as chromosome duplication and metabolite synthesis. The chromosome duplication process is, perhaps, a mere qualitative modification of this latter process mediated in part by cyclic pH changes. If the change in the nucleoplasmic pH accompanying the advent of the late interphase or the early prophase were in the direction of the pI's of the daughter metabolites and parent templets, the charge upon these particles and the consequent coulomb repulsion between them would be reduced. Then, as the synthesis of metabolite molecules proceeded, they would no longer be ejected from the chromosome but would remain adsorbed at their respective loci of synthesis.

Chromosome Structure and the Role of Nucleic Acid. While we do not have conclusive knowledge of the precise manner of association of the proteins that go to make up the chromosome thread, recent work has been very enlightening. It is known from the work of Mazia and Jaeger (1939), Mazia *et al.* (1941, 1947) and others (*cf.* Catcheside and Holmes, 1947) that the chromosome possesses a continuous protein framework that does not depend upon the presence of nucleic acid. Mazia interprets the results of his enzymatic studies to indicate that the chromosome possesses a fibrous structural or skeletal protein (digestible by trypsin but not by pepsin) which is different from the protein (digestible by pepsin) of which the matrix is composed, and he regards this interpretation to be supported by the ultraviolet absorption measurements of Caspersson (1939). The differential staining and ultraviolet absorption experiments of Fujii and Yasui (*cf*. Kuwada, 1937) have also differentiated a structural fiber from the matrix. Using the electron microscope, Buchholz (1947) has concluded that a continuous fiber extends the length of the chromosome and that individual genic granules (termed chromomeres[3]) are attached thereto. The recent work of Mirsky and Ris (1947) is especially significant. These investigators extracted isolated thymus lymphocyte chromosomes with 1M NaCl. A fraction of from 90 to 92 per cent. of the chromosome weight consisting largely of desoxyribonucleohistone (identified with basichromatin) was dissolved, leaving behind a coiled thread, the "residual chromosome." Mirsky and Ris concluded that the form of the chromosome is due primarily to the protein thread (definitely non-histone and identified with oxychromatin) of the "residual chromosome," and that the genes are either organized about or form a part of the substance of this structure.

It is well known that large quantities of DNA (desoxyribose nucleic acid) are associated with the chromosomes during mitosis and meiosis. Brachet (1944), using the pyronine-methyl green staining technique, has provided evidence that RNA (ribose nucleic acid) is present in some chromosomes, while Mirsky (1947) and Mirsky and Ris (1947) have shown directly that in liver chromosomes, thymus lymphocyte chromosomes and trout sperm nuclei, varying amounts of RNA are present.

Much recent work shows the intimate association of nucleic acid with protein synthesis (*cf*. Caspersson, 1947; Painter, 1945; Spiegelman and Kamen, 1946, 1947). Caspersson and his collaborators have been especially active in this field, and Caspersson (1947) recently stated that the evidence seems conclusive that in general all protein synthesis requires the presence of NA (nucleic acid). Thus, a primary function of the NA appears to be that of participating in the ancillary reactions of protein synthesis and the grosser aspects of chromosome duplication. As a result of studies of the last few years (Astbury and Bell, 1938; Brachet, 1947; Caspersson *et al.*, 1940, 1941, 1942, 1947; Darlington, 1942; Mazia *et al.*, 1939, 1947; Mirsky, 1943; Mitchell, 1943; Painter, 1945; Painter and Taylor, 1942; Pollister and Lavin, 1944; Schultz, 1941; Schultz *et al.*, 1940) a clearer picture of the NA metabolism is emerging. Thus, for example, Brachet (1940) showed that in the fertilized sea urchin egg there is far more RNA than DNA, but that as the egg develops (after fertilization) the DNA content of the nucleus rises and at the same time the quantity of RNA in the cytoplasm falls. This led Brachet to suggest that one type of NA is synthesized at the expense of the other. In this connection, washing a yeast culture removes most of the RNA of the cytoplasm without extracting the DNA from the nucleus, and yet after this treatment the cells grow when placed in a suitable medium (Delaporte, 1939). There is now conclusive evidence that RNA is also present in the nucleolus (Brachet, 1940; Caspersson and Schultz, 1940; Mirsky, 1943), which is known to be very intimately related to the chromosomes and chromosome activity. The present picture of the NA metabolism would appear to be somewhat as follows. The heterochromatin regions of the chromosome (*i.e.*, the nucleolus-associated chromatin) are believed to take part in the synthesis of DNA (and perhaps also RNA) and thus to act as a sort of regulating center for the NA metabolism of the chromosomes and the entire cell. The nucleolar apparatus appears to function in the transfer of NA between the nucleus and the cytoplasm, and to a large extent in the interconversion of the two types of NA. RNA, the only NA normally found in the cytoplasm, takes part in cytoplasmic protein synthesis. Thus, for example, in secreting cells (*cf.* Painter, 1945) there is a continual synthesis of

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[3] It seems desirable to abandon the term "chromomere" since Ris (1945) believes that "chromomeres" are either misinterpreted gyres of the chromonematic helix, points of overlap of chromonemata or heterochromatic sections where the chromonema is more tightly coiled. Ris points out that a gene represents a region of relatively considerable length along the chromonema

NA by the heterochromatin. This NA is converted by the nucleolus into products which are transferred to the cytoplasm to yield RNA exclusively. The RNA is in turn consumed in the cytoplasmic processes of synthesis of protein secretory products. Brachet's work shows that the reverse action (*i.e.*, cytoplasm to nucleus) also takes place, so that the NA metabolism may take on a cyclic character.

It seems evident, at any rate, that DNA (and possibly RNA as well) is closely associated with the processes of skeletal unification that serve to unite the synthesized proteins into the continuous chromosome framework. Thus, Schultz (1941) has suggested that a special function of the DNA might be in the actual synthesis of the fibrous proteins of the chromosome, a point of view that he believes to be in accord with the results of the x-ray analysis of Astbury and Bell (1938), and this is essentially the view expressed recently by Caspersson (1947) and Hydén (1947).

There is some evidence that genic duplication may take place more or less independently of the structural fiber–formation process of chromosome reproductive activity. This is provided by observations of the "lamp brush" chromosomes in the oöcytes of many vertebrates (Dodson, 1948; Duryee, 1941; Koltzoff, 1938). These chromosomes are large and extended and appear differentiated into genic granules that stand out sharply like a string of beads. The individual genic granules appear to undergo a series of longitudinal divisions in the egg nucleus, producing lateral branches and loops made up of granules like the original. These branch granules probably act as auxiliary synthetic centers, thereby temporarily increasing the capacity of the chromosomes for metabolite synthesis (*cf.* Dodson, 1948). At a later stage these lateral extensions are lost in the cytoplasm. It would appear that *fundamentally* it is only the lack of the longitudinal continuity of the structural fiber between these branch granules that distinguishes the formation of "lamp brush" chromosomes from chromosome duplication.[4]

The particles which are unified by the formation of the skeletal protein fiber appear to be those genic or subgenic proteins otherwise elaborated as metabolites (or the proteons of these metabolites). The separation of the parent and daughter[5] threads probably occurs somewhat after the manner suggested by Darlington (1937). Thus, changes in the plasm pH may affect an increased coulomb repulsion between the daughter centromeres and a waning of the polar centrosomic repulsion.

The pairing of chromosomes which occurs during meiosis appears to be caused by inherent variations in the nucleoplasmic pH–chromosome pI cycle and the consequent or determining NA attachment-detachment cycle during this process. One of the variations appears to be that during the early prophase the pH of the nucleoplasm and the pI of the chromosomes approximate one another more nearly than during mitosis. The other variation appears as a concomitant temporal one in which the meiotic prophase is initiated before the disassociation of chromatids and the resulting effective saturation of the attractive forces (see below) has taken place. The net result of these and other related variations seems to be that the coulomb repulsion between homologous chromosomes is very small during meiotic prophase so that the LRSAF between their genes and centromeres is able to bring them together.[6]

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[4] Painter and Taylor (1942) have pointed out that the granules of the side branches do not contain DNA, while Dodson (1948) has demonstrated the presence of RNA. From the functional point of view these observations are not surprising, since if DNA is chiefly concerned with synthesis of the fibrous proteins of the chromosome, its presence might not be essential in the side branches which apparently originate by duplication of only the mother granules of the chromosome proper.

[5] According to Darlington (1937), observations of the reproductive behavior of ring chromosomes appear to demand that there be an absolute distinction between a parent and daughter thread, rather than a division of the chromosome into two equivalent daughter threads. The synthesis of the daughter thread may occur one mitotic cycle (or more) in advance of the disassociation for evidence is accumulating that the anaphase chromosome consists of 2 or even 4 chromonemata. Thus, the duplicating activity of the chromosome might continue into the anaphase.

OTHER CYTOLOGICAL CONSIDERATIONS

Evidence for a Long-Range Specific Attractive Force. The evidence that a LRSAF acts in chromosome mechanics is quite convincing. At mitosis in the diploid of many Diptera (and of numerous other plant and animal species) homologous chromosomes are seen to lie unusually close together on the metaphase plate, with homologous parts lying parallel. The same somatic pairing has been observed between the homologous chromosomes of polyploid plants during mitosis. In this case, with more than two chromosomes being attracted to one another, the groups lie radially instead of parallel. A similar juxtaposition of homologous bivalent and univalent chromosomes (secondary association) has long been known to occur. The smaller, more mobile chromosomes are more likely to show somatic pairing than the larger ones (*cf.* Darlington, 1937; Fabergé, 1942, for a further discussion and references). Thus, a specific long-range attraction (like that which brings the chro-

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[6] It is apparent that the LRSAF must be presumed to act to a certain extent between all chromosomes, it being fully effective only in the case of homologues. This follows because of the essentially identical composition of chromatin, the differences, while apparently not great, being highly determinate. Moreover, the structural protein fiber of the chromosome may be of identical composition throughout chromosome complements. Thus, the equilibrium of the metaphase plate may be regarded as resulting (in the plane of the plate) from the equilibrium of interchromosomal forces of attraction and coulomb repulsion. The maintenance of the plate itself seems to reside largely in the strong repulsion between the centromeres and centrosomes. With respect to the above discussion (text) an inverse relationship appears to exist between the extent of association of the chromosome with NA and its negative coulomb charge. In meiosis the chromosomes are associated with greater amounts of NA than during mitosis and they also apparently carry a lesser charge. On the other hand, the centromere, which appears to be highly charged during anaphase movement, is not associated with NA to any noticeable extent (*cf.* Darlington, 1947). This relationship may have significance with respect to the nature of the bonding of the NA to the protein.

mosomes together at meiotic prophase) appears to be acting in somatic pairing and secondary association to modify the even equilibrium of the metaphase plate. Numerous experiments (Hinton, 1946; Muller and Painter, 1929; Van Atta, 1932) have shown that when chromosome aberrations exist, displaced segments tend to pair with their homologues, even though these segments may have been displaced to non-homologous chromosomes. The stretching and pulling out of position thus induced provides unmistakable evidence of this action. From studies of chromosomes undergoing somatic pairing Hinton (1946) concluded that the LRSAF may be effective at distances as great as 10 microns, and for purposes of calculation Fabergé (1942) uses 4 microns as an average distance at which leptotene chromosomes are separated at the beginning of pairing. That the LRSAF which secures chromosome pairing is specific gene for gene is shown by the pairing features in certain cells heterologous with respect to translocations, inversions and other aberrations. In these cells, the pairing of homologous genes occurs even though the chromosomes must be thrown into crosses, loops, etc., in order that this may occur (Belling, 1927; McClintock, 1933; Muller, 1941; Painter, 1934).

Saturation of Attractive Forces. Evidence that the attractive forces become effectively saturated by the association of chromosomes in pairs is provided by observations in several triploid (and tetraploid) plants (Darlington, 1937). On the triploid meiotic cells all three of the homologous chromosomes approach each other in the prophase, but pairing between them appears characteristically between two only in any given region. The third chromosome may be paired with either of the other two at other regions, or if the two are paired throughout their length, the third may remain free altogether, lying parallel to the bivalent. Saturation effects are also shown when chromosomes happen to divide before pairing is completed. In these circumstances the pairing process is brought to a halt, the divided segments failing to pair with one another (Darlington, 1937).

That the protoplasm does not attain the pI of the chromosomes, even in meiosis, is shown by the fact that there exist at all stages repulsions of various strengths between bivalent and univalent chromosomes. The initial pairing is accomplished because the LRSAF is able to overcome the minimal coulomb repulsion, and not because repulsion has ceased. Thus, after formation of the tetrad, the attractive forces are effectively saturated by the association of the sister chromatids of each dyad, so that the dyads are no longer held together. As a result of the coulomb repulsion these fall apart (in early diplotene), being held together only where chiasmata have formed or at the centromere.

II. FOREIGN PROTEIN-INDUCED PHENOMENA

We turn now to a generalized discussion of the action of foreign proteins. Consider the introduction of a foreign protein (non-nucleoprotein) into the tissues of an organism. Playing no part in the normal metabolism of the organism, the foreign protein would tend to disturb the delicately balanced dynamic equilibrium of the protoplasm. One effect would undoubtedly be the attraction and adsorption of certain subproteons by the foreign protein, in the same manner as do the genes. The synthetic activity of the genes is provided for by the proper ancillary mechanisms which are normally present in the protoplasm. In the case of a foreign protein, however, such ancillary synthetic mechanisms would not be guaranteed. Any synthetic activity of the foreign protein would depend upon the extent to which the normal metabolic processes of the host tissues could be utilized as ancillary synthetic mechanisms for its own duplication. Subproteons of the foreign protein similar to those subproteons normally synthesized by the host tissues in the formation of its own protein complement would be likely to exert synthetic activity. Thus, certain subproteons of the foreign protein (the antigen) could be expected to reproduce themselves, just as in the synthesis of metabolites by the genic templets (the synthetic activity of these subproteons would be specifically modified by the presence of haptens). If the protoplasmic pH at the site of synthesis were sufficiently distinct from the pI's of the reproducing subproteons (the *antigenic groups*), the synthesized molecules (the antibodies) would be elaborated, after the manner of metabolite elaboration by genes. Thus, the foreign protein would consume normal tissue components and synthesize and elaborate partial replicas of itself. It is unlikely that the organism would possess any adequate *direct* combative mechanism to obviate this derangement of its metabolism; however, the process might tend to check itself in the following way. In other tissues of the organism more isoelectric to the antigenic groups of the antigen, the LRSAF between these groups and the antibodies would be effective and would result in adsorption by the antigen of any of the antibodies encountered there. Thus, in these tissues, the antigen would form *primary complexes* with the antibodies. The increase in size due to this aggregation would result in a decreased brownian velocity of the primary complexes, permitting them to aggregate with one another under the action of the LRSAF to form *secondary complexes.* By this process inert aggregates would be formed. In this regard it is known that the antibodies, wherever they are produced, are rapidly distributed by the bloodstream and are in part fixed by various tissues (Topley and Wilson, 1946). These inert aggregates are probably degraded by the clearing organs or cells (both the macro- and the microphage engulf and digest formed antigenic material). This would automatically tend to prevent the synthetic activity of the antigen from completely disrupting the metabolism of the host.

If an additional amount of the antigen were introduced into the immune organism, the particles thereof would be rapidly inactivated, acting as they would (in certain tissues) as foci of adsorption for the antibodies present. It is presumably the same inactivation process that occurs *in vitro* when antibodies are brought together, under appropriate conditions, with the antigen. It is to be noted that the union of antigen with antibody merely results in inhibition of the synthetic behavior of the antigen through saturation of the LRSAF, and does not in itself act to destroy the properties of either component. Thus, for example, it appears that a more or less loose combination of toxin and antitoxin takes place, the poisonous properties of the toxin being held in abeyance as long as the union persists (Jordan and Burrows, 1942).

A single antigen could give rise to many antibodies, since it merely provides the synthetic templets. The size of the antibody would not necessarily be related to the size of the antigenic group or the antigen, since the antibody need not consist of a single proteon, but might be an aggregate of proteons. These proteons might aggregate (through hydrogen bonding) at the site of synthesis, or they might be brought together through the action of the LRSAF after ejection from the antigenic group. The feasibility of this latter type of action is borne out by Svedberg's (1940) work which shows that soluble proteins are often capable of reversible association and dissociation into particles whose molecular weights are usually some simple multiple or submultiple of that in the original phase. Thus, proteins may be regarded as reversibly dissociable component systems. The work of Svedberg (1940) and his coworkers has also shown that for a given protein the molecular weight as a rule has its maximum value within a given pH range around its pI. Svedberg (1938) concludes that synthesis of the protein particle is probably accomplished by the successive aggregation of definite units.

A certain amount of evidence indicates that antibodies are aggregates of proteons. Thus, in different species immunized, an antigen gives rise to antibodies whose molecular weights vary from 156,000 to 930,000 (Kabat, 1939). Furthermore, the larger antibodies are disaggregated by relatively mild treatment with barium hydroxide without much reduction of precipitating power (Topley and Wilson, 1946), and although antibodies have been found relatively resistant to the action of pepsin at pH's greater than 3, they undergo partial degradation without loss of antibody function (Schmidt, 1944). In addition, ultrafiltration experiments on horse and rabbit anti-pneumococcus serum have demonstrated the presence of aggregates which are dispersed by changes in the medium (Landsteiner, 1946).

It has been suggested that only those subproteons of the antigen that are closely related to subproteons or proteons of the normal host proteins can act as antigenic groups. This being the case, an essential similarity of all antibodies could be anticipated. Only those subproteons of the antigen greatly similar to proteons of *generally occurring* proteins would be able to manifest antigenic behavior. In this respect, analytical, ultracentrifugal and electrophoretic studies all show highly purified antibodies to be typical proteins, which are very closely related to one or another of the normal serum globulin fractions. A close similarity of antibodies to one another is clearly brought out by experiments in which antibodies themselves are used as antigens in other species (Landsteiner, 1946; Treffers, 1944). Thus, it appears that only those subproteons of the antigen which are closely related to the proteons of the serum globulins may manifest antigenic behavior. This would indicate that the serum globulins possess a rather characteristic distinction, inasmuch as they are pointed up as being similar between different species to the extent that their ancillary synthetic mechanisms are practically interchangeable. Of interest and perhaps significance in this respect is the work of Cannon *et al.* (1945) who fed various foreign proteins to protein-deficient rats. It was found that the best weight recovery and the most effective serum protein regeneration occurred in animals fed certain globulin fractions, while the albumins gave poorest response. These effects are probably related primarily to the content of essential amino acids[7] of the globulins (Cannon, 1945), but it is possible that globulins (which need not be digested to the amino acid stage) are most readily and efficiently converted to the proteins of the animals to which they are fed.

It has been found (Cohen and Chargaff, 1940) that thromboplastic protein-antibody precipitates are more active in promoting blood clotting than is the antigen contained in them, and that serological inhibition reactions can be obtained with proteoses (*cf.* Landsteiner, 1946). These findings provide rather indirect support for the contention that antibodies consist of proteons identical with certain subproteons of the antigen.

Experimental work has failed to disclose any *consistent* differences in the physical and chemical properties of antibodies and normal serum globulin. Consequently, it must be concluded that the proteons of the antibody and normal serum globulin, while differing serologically, are otherwise greatly similar. This serological distinction probably resides in differences in the composition and spatial distribution or configuration of antibody subproteons from those of normal serum globulin. Breinl and Haurowitz (1930) and Mudd (1932) have suggested that the ordering of the amino acids in the polypeptide chain of the antibody might be different. On the other hand, Rothen and Landsteiner (1939) pointed out that different ways of folding the same polypeptide chain might be involved, and this is essentially the view adopted by Pauling (1940). Practically the same situation is found to occur in the case of the highly potent soluble bacterial toxins, which do not seem to differ in any essential respect from bland proteins. The balance of evidence indicates that the toxicity is a property residing in the structure of the toxin molecules (Jordan and Burrows, 1942). In any case, the different behavior of normal and immune globulin toward the antigen would be accounted for, since the LRSAF appears to be highly dependent upon specificity of structure.

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[7] The essential role of the serum globulins is unknown but in view of their high content of essential amino acids and our conclusion that they are probably greatly similar between different species, the possibility suggests itself that they bear somewhat the same relationship to the amino acids of the blood as hemoglobin does to oxygen. Thus, the globulins and amino acids may form loose reversibly dissociable adsorption complexes, the equilibrium being governed by variations in the plasma characteristics induced by the state of the specific tissues encountered.

For the sake of simplicity the importance of the pH in the antigen-antibody aggregation process has been stressed. It is to be noted, however, that agglutination and precipitation *in vitro* usually do not occur in the absence of electrolytes. Yet it is only the macroscopic effect (formation of secondary complexes) that is lacking, for in both cases it has been shown (*cf.* Landsteiner, 1946; Topley and Wilson, 1946) that the antigen-antibody union (formation of primary complexes) takes place. This latter phenomenon serves to emphasize the real distinction between the initial formation of primary complexes and further aggregation leading to precipitation. The electrolytes appear to reduce the charge carried by the interacting particles, thereby reducing the pH sensitivity. It is known (Coulter, 1921; von Euler and Brunius, 1931) that pH changes affect the adsorption of antibody by the antigen, especially in the absence of electrolytes, although there appears to have been no systematic investigation of this factor. The above considerations of the antigen-antibody reaction have been patterned primarily after the aggregation of antigen with precipitins or antitoxins, where the brownian velocity is probably a significant factor.[8]

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[8] In the precipitin reaction, for example, the rate of precipitation is increased with an increase in temperature. However, the concomitant increase in the velocity of the particles reduces the probability that aggregation will occur and decreases the stability of the aggregates, so that beyond a certain temperature, dissociation of the antigen-antibody complex occurs. The formation of primary complexes is evidently the factor that brings the brownian velocity of the antigen down to the effective secondary aggregation zone, while increases in the temperature to values that do not exceed the upper limit for this zone can further enhance the flocculation.

(*To be continued*)

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ON CORRELATION OF THE PHENOMENA ASSOCIATED WITH CHROMOSOMES, FOREIGN PROTEINS AND VIRUSES

(Continued from March–April issue)

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III. VIRUS-ASSOCIATED PHENOMENA

CHARACTERISTICS AND REPRODUCTION

All viruses that have been examined are known to contain NA. Plant viruses are found to possess only the ribose type (but not identical with yeast NA), while both DNA and RNA have been found in animal viruses (*cf.* Beard, 1948). The molecular weights of viruses usually lie in the millions (up to several hundred million) so that they are generally much greater than those of proteins. Viruses are known to mutate, just as if composed of genic material. Over 50 strains of tobacco mosaic virus, for example, are known, indicating a considerable complexity of these bodies. Even the small foot-and-mouth virus (10 millimicrons in diameter) does not have a simple antigenic structure (*cf.* Daubney, 1934). Other direct and indirect evidence indicates that the high molecular weight plant viruses are built up from the union with NA of a number of similar proteins with more usual molecular weights (Bawden, 1943).

These considerations lead one to anticipate a similarity between the autosynthetic activity of viruses and that of genes and chromosomes. In this regard, the virus is essentially a large foreign protein which, so to speak, carries the basic mechanisms for its own NA metabolism with it. This is probably why viruses are usually extremely successful in their parasitism of a host. Since the vital NA is bound directly to the virus proteins, the virus requires only the ancillary cellular processes of the host which yield a suitable form of energy and the proper building blocks for proteins and NA.[9]

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The inability of viruses to multiply in cell-free media or in injured or dead tissues is undoubtedly related to the essentially endothermal nature of the reproductive process, which apparently can only utilize energy as provided by normally metabolizing protoplasm. By analogy with the phenomena of chromosome duplication, we are led to expect that the reproduction of the virus must take place in tissues, the pH of which is close to or within the isoelectric zone of the virus subproteons. This is because the synthesized subvirus units (the analogues of metabolites) probably must remain adsorbed to the synthetic loci if they are to become united in the formation of the daughter virus particle.[10] This point is of considerable significance. Thus, with the pH of the tissues in the isoelectric zone, and relatively constant (unlike during cell division), the parent and daughter virus particles would remain in association with one another. Hence, reproducing virus particles would form multiple associations or *primary aggregates*. Combination of these with one another would lead to the formation of *secondary aggregates* and precipitation. The formation of foreign bodies is actually observed in the course of most virus infections, and, indeed, it may be said that the most characteristic symptom of viral infection is the formation of the well-known intracellular inclusion bodies.

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[9] In much of the subsequent discussion an essentially pure nucleoprotein composition of the viruses is assumed. This is sufficient for our purpose, since it is the behavior of this material that primarily concerns us. For those viruses for which significant amounts of other constituents are known to be present, an activity modified in the direction of the activity of the simplest forms of bacteria probably obtains.

[10] The recent suggestion of Luria (1947) is of interest in this connection. On the basis of a statistical analysis of certain irradiation experiments, Luria suggests that the mechanism of reproduction of phage particles may consist of an independent reproduction of a number of subvirus units (as many as 50) which are thereafter incorporated into the final phage particle.

INCLUSION BODIES

Extensive experimental data warrant the conclusion that inclusion bodies are essentially aggregates of the infecting virus. These bodies can only be produced experimentally by the inoculation of infective viruses; they are not formed after inoculation of attenuated viruses, even though they have immunizing properties. Inclusion bodies are known to be closely related to the infectivity of the tissue. In herpetic keratitis of rabbits, for example, the disease becomes no longer inoculable with their disappearance (Topley and Wilson, 1946).

The inclusion bodies vary morphologically from what are apparently true crystals to hyaline particles and amorphous granules of various characteristic and irregular forms. It is likely that this variable morphology is determined by such factors as the pH, metabolic state and viscosity of the infected cell protoplasm, the size of the virus, the extent of primary aggregation that takes place before secondary aggregation commences and the point of saturation of the attractive forces.

It is well known that certain plant and animal virus diseases produce no characteristic visible inclusion bodies, and this may be attributed to variations of the above factors which inhibit the formation of large aggregates. The evidence clearly shows that the absence of visible inclusion bodies can be as much a characteristic of the infected tissue as of the virus itself (*cf.* Bawden, 1943; Lipschütz, 1925; Topley and Wilson, 1946).

Descriptions of the process of inclusion body formation in plant and animal virus infections have been given by Sheffield (1931) and Ludford (1928). The first definite sign of infection is the appearance of minute granules in the cytoplasm. With the progress of the infection these increase in number and size, fusing together as they come in chance contact with one another. This process ultimately results in the formation of one or more large inclusion bodies. The destruction of these bodies is doubtless a direct action of intracellular clearing mechanisms coupled, in animals, with the clearing organs and phagocytes. It is the aggregation of the virus into these inclusion bodies and the consequent complete loss of synthetic activity that permits the clearing mechanisms to gain ascendency over them. If the fate of plant chromosomes which through lagging are accidentally lost in the cytoplasm is considered, there can be little doubt of the action of intracellular clearing mechanisms in plants. In the triploid *Lilium tigrinum,* for example, it has been observed that chromosomes which lag and are left in the cytoplasm upon formation of the nuclear membrane form micronuclei or microcysts which eventually degenerate (Chandler *et al.,* 1937). In view of this action it is not unreasonable to suppose that a similar destruction of intracellular inclusion bodies, which are *foreign* substances, occurs.

FURTHER ASPECTS OF VIRUSES AND VIRUS INFECTION

In general the pathology of virus diseases runs the gamut of the possible types of tissue injury (Rivers, 1928). This fact alone indicates an extremely varied apparatus of pathogenicity. Of course, it is reasonable to attribute a great deal of the variation to a difference in the metabolites and other tissue constituents depleted by the same or different viruses in different hosts. In this connection the studies of Caspersson (1947) and Hydén (1947) indicate that viruses interact with the host cell as parasites on the system for protein production, different viruses intruding upon different phases of this system. However, many other factors are probably determinate. In certain tissues, for example, the virus is undoubtedly incapable of reproducing due to the lack of certain essential subproteons. This is not unlikely in view of the fact that in the same organism different tissues frequently contain quite distinct proteins and may vary to a considerable extent in their amino acid content. Certain evidence points up the fact that viruses generally prefer tissues in which growth metabolic processes are in action. This is to be anticipated in view of the nucleoprotein constitution of the viruses. Thus, it is found that the effect of the virus on plants showing mosaic symptoms is one of significantly reducing the assimilatory tissues. Moreover, only the younger leaves show symptoms. Regarding animal viruses, Duran-Reynals (1928, 1929) has called attention to the enhancing effects of extracts of embryonic or sarcoma tissues; and other evidence shows that animal viruses prefer young newly formed cells for their multiplication.

The formation of inclusion bodies and primary aggregates probably acts as an automatic checking mechanism to the further course of the disease, since the effect of aggregation is to decrease and finally saturate the LRSAF, as well as to limit the synthetic activity to the outermost particles of the aggregate. The latter type of effect is not infrequently encountered in experimental work. For example, treating tobacco mosaic virus with acids or salts appears to increase the size of the infecting units by causing aggregation of the virus particles, and so reducing the infectivity of a given weight of the virus. In certain dilution experiments with plant viruses it is found that the relative infectivity increases in a manner indicating a dissociation of aggregates of virus particles into smaller infective units (*cf.* Bawden, 1943).

In cases where the reproduction of viruses is not accompanied by secondary aggregation and the formation of inclusion bodies it is to be anticipated that the reproductive process will be of a more sustained autocatalytic character inducing more severe symptoms. This is confirmed by the observation that a virus may produce many inclusion bodies in all plants in which it causes mosaic symptoms, but *it is unusual for it to produce them in hosts in which it causes severe necrosis* (*cf.* Bawden, 1943). It is also found that in the conversion of rabies " street virus " to " fixed virus," *the* *ability to produce Negri bodies is lost coincidentally with the development of increased virulence* (*cf.* Jordan and Burrows, 1942), and, instead, very fine granules may be found in enormous numbers in the nerve cells (Manouélian, 1912).

It is an essential characteristic of the antigen-antibody aggregation process that it does not result in the destruction of the antigen or antibodies but merely in a mutual inactivation. A large body of evidence supports this conclusion, for by the appropriate treatment of the antigen-antibody complex, either the antigen or the antibody can often be recovered with all its original properties (*cf.* Bawden and Pirie, 1937; Chester, 1936; Landsteiner, 1946).

Some direct observational evidence of the serological reaction *in vitro* recalls to mind the process of inclusion body formation. Thus, if a plant virus is mixed with its antiserum (prepared by inoculation of animals with the plant virus) and the mixture kept circulating by convection currents to simulate cytoplasmic streaming, small particles are seen to appear throughout the fluid. These fuse upon coming together so that large aggregates, which settle out of solution, are formed (Bawden, 1943). It is evident that the antibody is effecting a mutual aggregation with the virus particles, which phenomenon *in vivo* halts the virus's autosynthetic activity. The agglutination of elementary bodies by immune serum may be observed in the case of the larger animal viruses both microscopically and macroscopically in the same manner as the agglutination of bacteria (Craigie and Wishart, 1934).

It has been noted that the plant virus particle appears to be built up from the union with nucleic acid of a number of similar proteins. The antigenic complexity of viruses is indicated by the large number of strains of various plant viruses and phages that are known. In the case of animal viruses, recent work has revealed an antigenic complexity similar to that present in many bacteria (Topley and Wilson, 1946). Direct evidence that the antiserum of a given virus is a mixture of different antibodies, and that each antibody is specific to a different antigenic group thereof.

is forthcoming from a large number of serological experiments with plant virus strains and their antisera and will not be detailed here (*cf.* Bawden, 1943). It is also noteworthy that, while related strains of plant viruses react strongly with one another's antisera, little or no effect is elicited by an unrelated strain.

Two kinds of inactivation of plant viruses have been observed. In one of these the loss of infectivity is accompanied by a denaturation of the virus proteins. Such treatment destroys the serological activity of the virus and its ability to crystallize or form liquid crystalline solutions. Other less drastic treatments render preparations non-infective without actually denaturing the virus proteins, and the serological reactions, crystallinity, and in some cases the antigenicity (*cf.* Bawden, 1943), are left unimpaired. This fact, *i.e.*, that the antigenicity of a virus may not be destroyed by certain treatments which render the virus itself non-infective, is directly in line with the interpretation that the infectivity and antigenicity of viruses are different phases of virus action. This seems also to be borne out by the fact that, while antisera against plant viruses may be prepared by animal inoculation, the plant virus is essentially non-infective against the animal. Treatments which render a virus non-infective (*i.e.*, unable to reproduce) without affecting its antigenicity probably affect the virus in such a manner as to directly or indirectly prevent the virus's NA from taking part in the metabolism of virus reproduction. Since NA appears to be vitally concerned in the processes that serve to unite the genes into the continuous chromosome framework, it is, perhaps, the analogous process of unification of subvirus units that is prevented.[11]

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[11] Some recent work of Caspersson (1947) and Hydén (1947) is of interest in this connection. These investigators have shown that in the reproduction of the more complex viruses a DNA mechanism is in play, *i.e.*, the general NA mechanism for protein synthesis and the specialization thereof for the reproduction of several proteins linked together—the chromosome mechanism. It is proposed that, being more complex, the higher viruses possess the DNA mechanisms providing for the exact linking of their many

On the other hand, antibody elaboration can occur independently of virus reproduction, for the ancillary NA metabolism of the cells is utilized, just as in the case of antibody production by non-nucleoprotein antigens. Consequently, even in the modified condition, the virus appears to be able to carry on this more elementary form of synthetic activity. Schramm (1941) has shown that enzymatic removal of NA from tobacco mosaic virus destroys its infectivity without any distinct change in the precipitin reaction with rabbit antiserum. From the functional point of view, this tends to bear out the contention that the role of the NA of the virus is essentially one ancillary to virus reproduction, and largely independent of virus antigenicity.

ANTIVIRAL IMMUNITY

The extension of the considerations of earlier sections leads to the following picture of the phenomena of antiviral immunity.

The virus reproduces in tissues of the body having a pH relatively isoelectric to the virus particle and its templet subunits. Reproduction occurs at the expense of the host's metabolism and usually leads to the formation of visual inclusion bodies, which, being inert and immobile, are eliminated by clearing mechanisms. In slightly less isoelectric tissues the greater coulomb repulsion between primary aggregates prevents secondary aggregation. These primary aggregates also tend to be eliminated by clearing mechanisms, but, being more mobile, some of them reach non-isoelectric tissues. Here they are disintegrated by coulomb repulsion (just as the volutin granules break up) into primary infective units which again invade susceptible tissues, securing the continued course of the infection and establishing the typical secondary foci of infection.

proteins, thereby insuring their proper distribution to the daughter individuals. Caspersson and Hydén believe that in the simplest viruses there is no need for such a mechanism so that only the RNA mechanism is present.

On the other hand, virus reproduction can not occur in tissues of the body non-isoelectric to the virus's antigenic groups. Instead, a synthesis and elaboration of subvirus units, the antibodies, probably occurs through a process analogous to metabolite synthesis and elaboration by chromosomes. In this regard it has been observed that the virus can frequently be demonstrated in other parts of the body even when the lesions themselves are confined to one tissue (Topley and Wilson, 1946) and secondary infections of tissues showing no primary symptoms occur frequently (Jordan and Burrows, 1942). The antibodies, which are rapidly transported by the bloodstream, act both to check established infections and to prevent new infection of the immune organism by forming inert complexes with the otherwise synthetically active virus particles. Formation of antigen-antibody complexes probably occurs only in isoelectric tissues, for here the LRSAF is able to effect an adsorption of the antibodies to the antigenic groups of the virus. In the absence of antibodies the virus particles would reproduce in these tissues (if other conditions also favored reproduction), thereby initiating the course of the infection. If antibodies are present, however, they are rapidly adsorbed, instead of the normal tissue components that would have been depleted in their absence.[12] The inert complexes formed are destroyed by the clearing mechanisms and by this means infection is obviated.

The Persistence of Antibodies. The effective and lasting immunity often conferred by a single attack of an animal virus is accompanied by a persistence of antibodies in the host's tissues. Immunity conferred by non-nucleoprotein antigens, although generally of much shorter duration, also involves the persistence of antibody in the immune animal. Offhand one might be inclined to suppose that the antibodies are able to persist because they are "masked" by their great similarity to the serum globulins. However, the serum globulins themselves, together with practically all other body proteins are known to be continuously in a state of synthesis and disintegration. Tracer studies indicate that the antibodies undergo the same rapid turnover as do the immunologically inert serum proteins (Schoenheimer, 1942), while it is evident from other immunological studies that the circulating antibody is being destroyed at a rate which is approximately proportional to its concentration (*cf.* Burnet, 1941). Moreover, it is known that immunized animals can suffer extensive bleedings without any detectible and lasting fall in antibody titre. Hence, the mechanism which secures the persistence of antibodies must be a more profound one. It would appear that one of the following three processes secures this maintenance.

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[12] However, in the case of antitoxic immunity, for example, the effectiveness of the antitoxin depends to a great extent upon its ability to intercept and neutralize the toxin *before* it reaches susceptible tissues. This is in accord with previous considerations of inactivation of non-nucleoprotein antigens.

1. The antigen persists throughout the immune state, serving continuously as a templet for antibody production.
2. The antigen effects a persisting modification in the apparatus for globulin production
3. Antibodies are self-duplicating.

The first alternative has formed the basis for the hypotheses of antibody formation by Breinl and Haurowitz (1930), Haurowitz (1937), Alexander (1931), Mudd (1932), and Pauling (1940). These investigators have assumed that a templet action of the antigen is the only method of antibody production that is involved in the immune animal. Hence, in the light of present knowledge one must interpret these hypotheses as ascribing antigenic templet action to account for both the initial formation and the continued persistence of antibodies. Thus, the antigen must be presumed to be present throughout the immune state. The persistence of the antigen in the face of constant degradative action, however, poses the same problem as does the persistence of the antibodies. Moreover, upon the basis of immunological evidence the general persistence of the antigen throughout the immune state must be ruled out. Practically all the indirect evidence supports the view that the antigen is destroyed by the organism long before it loses its capacity to produce antibody, so that the supposition that the antigen persists unmodified, but undetectible by any experimental methods, could only be adopted if no other interpretation could account for the facts. Conditions of apparent antiviral infection-immunity, in which the virus is known to be present in the immune animal long after infection, are known. However, in such cases there is always clear epidemiological evidence of the persistence of the infection. These conditions certainly do not occur in measles, yellow fever, etc., so that it is difficult to avoid accepting long-lasting antiviral immunity as something induced by the infection, but maintained by the organism after all the virus has been destroyed (Burnet, 1941; Francis, 1947).

Additional evidence that the continued production of antibodies is not secured by a templet action of the antigen is provided by studies of the rate of increase of circulating antibodies. It is clear from these studies that an autocatalytic process is somewhere involved for a time (Burnet, 1941), and this could not be secured by a non-reproducing antigen, whose antibody production would proceed more or less linearly.

Thus, while the hypothesis that antibodies are initially produced by a templet action of the antigen is acceptable,[13] it seems evident that the persistence of antibodies in the immune condition can not be reasonably attributed to the same mechanism.

The second alternative is the one adopted by Burnet (1941). According to his hypothesis the contact of the antigen with the so-called proteinase securing globulin production results in its modification, perhaps in such a manner that it can effectively degrade the antigen. The modified proteinase retains its modification during subsequent production of complete or partial replicas (the antibodies or their precursors) but in the absence of continued stimulation by the antigen the apparatus gradually reverts to normal globulin production. Burnet argued this hypothesis by analogy with that for the formation of new "adaptive" enzymes for certain substrates by bacteria. In the light of recent work (*cf.* Sevag, 1946; Spiegelman, 1946), however, it seems likely that the system for the production of "adaptive" enzymes occurs normally in a more or less non-functional condition. Hence, this argument now appears less convincing.

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[13] In the case of the production of antibodies against tobacco mosaic virus in mice, the tracer studies of Libby and Madison (1947) indicate that the virus must be present to stimulate the formation of antibody, for there appears to be a coincidence in the time between the breakdown of the virus and the regular decrease in the amount of circulating antibody. Thus, it appears that in the atypical case of antibody production against plant viruses injected into animals, templet-acting antigens may be primarily involved.

The second alternative also raises the question of the manner of persistence of the modified globulin-producing apparatus in the face of constant degradative action. It is presumably the action of genotypic factors[14] (*cf.* Beadle, 1946) that normally guarantees the synthetic processes which equilibrate with degradative processes to preserve the integrity of the phenotype. In the absence of these factors the degradative processes would eliminate the modified apparatus and so terminate the production of antibody.

On the other hand, it is possible that the evidence for a dynamic equilibrium is interpreted too strongly and that degradation is limited to certain systems only, or in many cases acts in only a superficial manner. Also, if the antibody-producing proteinase apparatus is self-reproductive, as Burnet has postulated, this factor might be sufficient to secure its persistence even though it competes with the apparatus for normal globulin synthesis. Thus, the second alternative can not be ruled out as a possible basis for the persistence of the immune state.

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[14] Of particular interest in this regard are the systematic immunogenetic studies of Cumley and Irwin (1942). The serum of doves and pigeons and their hybrids was investigated. These authors showed that the species hybrids possess the proteins of both parent species, and that these proteins segregate in backcross hybrids. It is concluded that beyond a reasonable doubt the proteins are controlled by gene action.

The third alternative offers the simplest and most direct mechanism for antibody maintenance and it has been suggested by Jordan (1940). In the light of the previous considerations an autosynthetic activity of antibodies is to be anticipated. The chief criticism against this alternative might stem from the fact that passive bacterial and antiviral immunity is of relatively short duration. Thus, if antibodies are capable of self-reproduction, injection of immune serum into the bloodstream would be expected to produce an effective immunity. However, it is well established that antibody production is a function of the tissues (or cells) of an organism and does not occur through a multiplication of antibodies in the blood plasma. It is known that labeled dietary nitrogen is not taken up by passive antibody, in pronounced contrast to the appearance of labeled nitrogen in active antibody (*cf.* Heidelberger, 1946). If antibodies are self-reproductive, this last observation indicates that humoral antibodies are not able to enter the cells in which antibody synthesis occurs. Thus, it is probable that the relatively short duration of passive immunity results because the injected antibodies are unable to become established at sites suitable for autosynthesis, whereas the active antibodies of infection *originate* at these sites.

Long-lasting antiviral immunity must find its basis in the enormous numbers of virus-antigens present (due to autosynthesis) and their marked intracellular parasitic habit. Thus, for example, in the early stages of yellow fever there is an extremely high content of virus in the blood, as much as ten billion infective doses per cc. (Hughes, 1933). This high concentration of virus of marked intracellular habit results in antibodies becoming established throughout the antibody-elaborating cells of the organism. This is essentially the view adopted by Burnet to account for lasting immunity, and it is generally supported by clinical evidence.

It will be noted that in the previous considerations of immunological phenomena and in the latter alternative, non-nucleoprotein foreign proteins are assumed to act synthetically or autosynthetically. Such an action is reasonable in view of the recent data which clearly show the mobility of the NA in the cell.

Indirect evidence that the antibodies act autosynthetically is provided by the observation that over certain periods in the establishment of the immune state, the rate of antibody production often appears to be roughly proportional to the amount of antibody present in the blood at any instant (*cf.* Burnet, 1941). Antibodies do not multiply in the blood plasma so this observation must be a reflection of the condition prevailing at the cellular or tissue-sites of antibody production. From this it may be inferred that over the period involved the antibodies are probably reproducing autocatalytically at these sites.

Although the third alternative includes the mechanism of the first within its framework, it is to be noted that *fundamentally* both the initial formation and the ultimate maintenance of antibody have been ascribed to the same mechanism, namely, the autosynthesis of antigen subproteons. The only distinction lies in the fact that these subproteons are presumed to act initially in their native setting, the antigen proteon.

It has been assumed that antibody production involves a synthesis of *new globulin* rather than a directed modification of the normal serum globulins. This is essentially the point of view adopted in most current hypotheses, and it appears to have satisfied both the chemist and the immunologist (*cf.* Schmidt, 1944; Topley and Wilson, 1946). With regard to this matter, the simplest interpretation of the data would regard the replacement of both normal and immune serum globulins as secured by a similar mechanism. This is the superficial appearance presented in bleeding phenomena, since bleeding appears to act as a stimulus to the production of antibodies in the same sense that it acts as a stimulus to the production of the serum proteins in general; *i.e.*, the induction of an active response tending to make good the loss sustained (Topley and Wilson, 1946). Furthermore, on immunization there is an increase in the globulin present in the serum (the total serum protein may increase as much as 15 per cent.), although not all the new globulin has in all cases been shown to be antibody (Jordan and Burrows, 1942; Landsteiner, 1946). This seems to call for an actual synthesis of immune globulins rather than a mere modification of the normal serum globulins.

The Site of Antibody Synthesis. The above considerations lead one to suspect that the synthesis of antibodies occurs at the same tissue-sites where the normal serum globulins are synthesized. Sabin (1939) has presented evidence that phagocytic cells of the liver and other tissues may give rise to normal serum globulin through the shedding of their surface films. In the presence of an antigen, characteristically different globulins, namely the antibodies are apparently produced. It is known that particulate antigens introduced into the tissues are largely dealt with by the cells of the lymph nodes draining the site of injection (in which case an early appearance of antibody is noted at the lymph nodes), while cells of the spleen, liver and bone marrow are chiefly concerned in the disintegration of antigens which reach the bloodstream. Numerous experiments (*cf.* Topley and Wilson, 1946) have shown that removal or severe injury of tissues containing those cells of the reticulo-endothelial system which are known to remove injected antigenic material from the blood and tissue fluids interferes with the production of antibodies. These observations and other facts have led many investigators to conclude that antibodies are formed in these phagocytic cells (*cf.* Breinl and Haurowitz, 1930; Burnet, 1941; Jordan and Burrows, 1942; Perla and Mormorston, 1941; Topley and Wilson, 1946). On the other hand, a great deal of evidence also supports a lymphocytic theory of antibody formation (*cf.* Bunting, 1925, 1938; Ehrich and Harris, 1945; Rich, 1944). In a recent paper, Ehrich and Harris (1945) have concluded that both the new observations and the old facts seem to fit into a theory of antibody formation in which the lymphocytes, as well as the macrophage, play an essential role. The recent findings of White and Dougherty (1946) regarding the manner of distribution of antibodies are of great interest in this connection. These investigators have concluded that in the normal dissolution of lymphocytes, serum globulins are released, and that in the immune animal the same process also releases immune globulins.

Plant Immunity. The immunity phenomena so characteristic of animals is never observed in plants and there is no evidence that antiviral antibodies are produced in plants. The bacterial diseases of plants and animals differ in essentially the same manner so that it is likely that characteristic differences between these forms of life form the basis for this difference.

One of the characteristic differences between plant and animal virus infections is that the plants usually contain active virus for as long as they live. Thus, it has been found that the plant acquires the disease and passes through the usual stages, and that after the symptoms have faded the virus is still present and fully infective to a fresh plant. On the other hand, the recovered plant is immune to further infection. Furthermore, this plant is usually immune to other strains of the same virus although not to other viruses. It seems probable that the recovery is mainly due to the checking effects of X-body formation and the formation of primary aggregates. It has been noted that both of these actions probably tend to halt the autocatalytic reproductive behavior of the virus, and this is evidently suppressed to a degree which permits practically complete recovery of the plants. It seems evident, however, that the clearing mechanisms of the plant are inadequate to eliminate the virus, since this is a rare occurrence.

The immunity of plants to further infection by a virus or its related strains may be accounted for in the following manner. All the virus present in the recovered plant appears to be in the form of primary aggregates, since X-bodies are not found. These, of course, greatly reduce the infectivity of the material present for reasons which have already been discussed. Additional virus introduced into the immune plant does not cause infection, since it is immediately adsorbed by the primary aggregates, thus, merely adding to the amount of the virus present in these aggregates. The fact that immunity from related strains is also conferred probably has its basis in the large number of antigenic groups common to various strains of the same virus. The variations that produce related strains do not appear to be essentially different from mutations, and it is known that the pairing of chromosomes is not noticeably affected by the presence of a large number of heterozygous genes. On the other hand, the plant is not immune to unrelated strains since they possess different antigenic groups and thus, no appreciable attractive forces are in operation between the primary aggregates and the unrelated virus particles.

It has been observed that the efficiency of one strain in preventing the multiplication of another appears to depend upon its ability to multiply and occupy the plant fully, for protection is restricted to areas in which the first strain has become fully established. This is in accord with the view that it is primarily the presence of primary aggregates of the established strain that confers the immunity and not merely an adjustment by the plant. It is also found that if related strains are inoculated together, both multiply, although they tend to settle in different parts of the leaf, and that mixed extracts of unrelated strains do not inactivate one another.

THE pH FACTOR

If the pH of virus preparations is lowered below their pI's, the viruses are denatured and lose their infectivity completely and irreversibly. Bacteriophage, also nucleoprotein in constitution, is instantly denatured and inactivated at pH's below 4.7 (Northrop, 1938). Most phages appear to carry a negative charge within the pH range over which they remain active, in this way resembling viruses and bacteria. Chromosomes[15] are also negatively charged, so that in general nucleoproteins appear to be unstable at pH's appreciably below their pI. It has already been noted that the negative charge of the chromosomes appears to be inversely related to the amount of NA associated with them, so that this instability and loss of infectivity may be partly due to effects on the bonding of the NA to the protein.

All the plant viruses for which values have been obtained have pI's from 3 to 5 (Bawden, 1943). This straddles the pH range of 3.9 to 4.7 which Yamaha (1935) has found for meiotic plant protoplasm. On the other hand, Small (1929) in summarizing numerous earlier data has concluded that " the reactions of plant cytoplasm must lie somewhere between pH 5.2 and pH 6.2, with little or no variation beyond these points, but with probable variations within that range." More recently Chambers (1940) has found the pH values for many plant and animal cells to be 6.7 to 6.9 for the cytoplasm and 7.6 to 7.8 for the nucleoplasm. However, the study of protoplasmic pH's is not in a very satisfactory state (*cf.* Heilbrunn, 1943) so that protoplasmic pH determinations can not as yet be accepted as conclusive, quite apart from the question of their significance. Moreover, the pI's of proteins as determined *in vitro* may only be considered as a qualitative indication of their pI's *in vivo.* It is not unlikely that the proteins exist in a somewhat different state *in vivo* than *in vitro* and this appears to be as true for plant viruses as for other proteins (*cf.* Bawden, 1943; Bernal and Fankuchen, 1941). Small (1929) has emphasized that in the living cell the proteins are almost sure to exist in a variety of complexes, and that these might exhibit a large number of partial equilibrium points and would tend to show isoelectric zones rather than sharply defined points.

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[15] An *in vitro* determination of the pI of the giant chromosomes of *Sciaria coprophila* by Churney (1941) yielded a provisional value of 3.3 to 3.6. Using the same technique, Duryee (1941) has found the pI of frog chromosomes to be 4.5.

In the case of animal viruses the pI's are found to lie in a far more alkaline range, generally in the neighborhood of 7.6 (Topley and Wilson, 1946). However, most of the animal virus pI determinations have been carried out in the presence of tissue proteins. In contrast, the pI of a relatively pure suspension of vaccinial elementary bodies was found to be between 4.3 and 4.6 (Beard *et al.*, 1938). This indicates that the animal virus pI determinations are in a too alkaline range as a result of the presence of tissue proteins. This result also indicates that the pI of the chromosomes is greater *in vivo* than *in vitro* which is more in accord with these considerations. While it has been assumed that the isoelectric zone of viruses, chromosomes, etc., is approached or even attained in the course of the phenomena that have been discussed, it should be emphasized that *changes* in coulomb repulsion are the essential concern, it being more or less beside the point whether the coulomb charge is ever reduced to zero.

The fact that the pI's of plant viruses lie in a far more acid range than do those of animal viruses tends to indicate a greater acidity of plant protoplasm and there are other considerations that support this view. The non-infectivity of plant viruses against animals may be due to the greater alkalinity of animal tissues, inasmuch as plant viruses may not encounter isoelectric tissues in the animal, so that reproduction is not possible. On the other hand, the same factor would facilitate the antigenic action of the animal-inoculated plant virus, for the production and elaboration of antibodies probably can only occur in relatively non-isoelectric tissues.

Conclusions

Phenomena associated with chromosomes, foreign proteins and viruses are discussed. The chief factors that appear to determine the course of these protein-associated phenomena are: the action of long- and short-range specific attractive forces, the saturation of these forces through particle association, coulomb repulsion, the brownian velocity of the particles, the pH of the protoplasmic medium, the isoelectric points of the particles concerned and the availability of energy (presumably from nucleic acid metabolism) and building blocks from ancillary processes.

The following relationships are suggested:

It is the action of a long-range specific attractive force which, in conjunction with specific short-range attraction, brings about the adsorption of the building blocks for gene and metabolite synthesis to the templet-sites of synthesis in the chromosome. The elaboration or ejection of the metabolite molecules from the templets at which they are synthesized, occurs as a result of the coulomb repulsion between the templets and the synthesized metabolite molecules.

In the duplication of chromosomes, and in metabolite synthesis by chromosomes, essentially identical substances are produced. In the former process, pH changes accompanying cell division reduce the coulomb repulsion so that these substances remain adsorbed to the chromosomic templets and become united to form a daughter chromosome. In the latter process the pH is such that the synthesized particles are automatically ejected from the sites of synthesis.

Antigens utilize normal metabolic processes of the host's cells to synthesize partial replicas of themselves, these forming the antibodies. The elaboration of the antibodies from the antigenic templet-sites of synthesis parallels metabolite elaboration. The antigen can only effect the synthesis of those of its subunits which resemble subunits of the host's proteins, since the ancillary synthetic mechanisms of the host are too specific to be converted to the synthesis of foreign subunits.

The role of antibody in recovery and immunity depends upon an aggregation reaction with the antigen, leading to the formation of synthetically inert complexes which are eliminated by clearing mechanisms. The aggregation of the antigen with antibody is brought about by long-range specific attractive forces and occurs at sites where the protoplasmic pH induces lesser coulomb repulsion between these particles than exists at the sites of antibody synthesis and elaboration.

Antibodies are capable of self-reproduction, so that once formed they can persist independently of the antigen. The immune state wanes as normal globulin synthesis displaces the competing process of antibody autosynthesis. Antibody production by both nucleoprotein and non-nucleoprotein antigens and antibody self-reproduction take place in the globulin producing cells.

The self-reproductive capacity of viruses is the result of the possession of a nucleic acid complement sufficient to insure the ancillary synthetic mechanisms for protein synthesis which parasitism on the host's metabolic processes does not provide. The reproduction of viruses is confined to tissues where the synthesized subvirus units are not elaborated from their templets but remain adsorbed to become united in the formation of the daughter virus particle. Because of this the daughter particle remains in association with the parent virus, and the continuation of this process leads to the formation of larger aggregates. These aggregates are the well-known inclusion bodies of viral infection. The inclusion bodies are synthetically inert since the long-range force of attraction becomes saturated in the large aggregate so that building blocks for further synthesis are no longer adsorbed.

The relatively lasting immunity conferred by viral infections results because the antibodies become established throughout the globulin producing cells. Passive immunity is of relatively short duration, since the injected antibodies are unable to become established in cells suitable for autosynthesis, whereas the active antibodies of infection originate at these sites.

Plant immunity is conferred by the persistence of the virus in relatively inactive aggregates. New virus gaining access to the plant is adsorbed to these aggregates under the action of specific attractive forces and inactivated by the association.

LITERATURE CITED

Alexander, J.
1931. *Protoplasma,* 14: 296–306.
Alexander, J., and C. B. Bridges
1928. "Colloid Chemistry," Vol. II. Reinhold Pub. Corp.
Astbury, W. T., and F. Bell
1938. *Nature,* 141: 747–748.
Bawden, F. C.
1943. "Plant Viruses and Virus Diseases." Chronica Botanica Co.
Bawden, F. C., and N. W. Pirie
1937. *Proc. Roy. Soc.,* 123B: 274–320.
Beadle, G. W.
1946. "Currents in Biochemical Research," 1–11. Interscience, New York.
Beard, J. W.
1948. *Jour. Immunol.,* 58: 49–108.
Beard, J. W., Finkelstein, H., and R. W. G. Wyckoff
1938. *Jour. Immunol.,* 35: 415–425.
Belling, J.
1927. *Jour. Genetics,* 18: 177–205.
Bernal, J. D.
1940. *Pub. No. 14, A. A. A. S.,* 199–205.
Bernal, J. D., and I. Fankuchen
1941. *Jour. Gen. Physiol.,* 25: 111-165.
Botta, B.
1932. *Arch. exp. Zellf.,* 12: 455–464.
Brachet, J.
1940. *Compt. rend. soc. biol.,* 133: 88–90.
1944. "Embryologie chimique." Masson et Cie. Paris.
1947. *Symposia Soc. Exptl. Biol.,* 1: 207–224.
Brandt, K. M.
1941. *Protoplasma,* 36: 77–119.
Breinl, F., and F. Haurowitz
1930. *Z. physiol. Chem.,* 192: 45–57.
Buchholz, J. T.
1947. *Science,* 105: 607–610.
Bunting, C. H.
1938. "Handbook of Hematology," Vol. I. Hoeber, New York.
1925. *Wisconsin Med. Jour.,* 24: 305–311.
Burnet, F. M.
1941. "The Production of Antibodies." Macmillan and Co., Ltd., Melbourne.
Cannon, P. R.
1945. *Advances in Protein Chem.,* 3: 135–154
Caspersson, T.
1939. *Chromosoma,* 1: 147–156; *ibid.,* 1: 562–604.
1947. *Symposia Soc. Exptl. Biol.,* 1: 127–151.
Caspersson, T., and K. M. Brandt
1940. *Protoplasma,* 35: 507–526.
Caspersson, T., Landstrom, H., and L. Aquilonius
1941 *Chromosoma,* 2: 111–131.

Caspersson, T., and L. Santesson
1942. *Acta Radiol., Suppl. 46,* 1–105.
Caspersson, T., and J. Schultz
1940. *Proc. Nat. Acad. Sci.,* 26: 507–515.
Caspersson, T., and B. Thorell
1941. *Chromosoma,* 2: 132–156.
Catcheside, D. G., and B. Holmes
1947. *Symposia Soc. Exptl. Biol.,* 1: 225–231.
Chambers, R.
1940. *Pub. No. 14, A. A. A. S.,* 20–30.
Chandler, C., Porterfield, W. M., and A. B. Stout
1937. *Cytologia,* Fujii Jub. Vol: 756–784.
Chester, K. S.
1936. *Phytopath.,* 26: 949–964.
Churney, L.
1941. "Cytology, Genetics and Evolution," 113–128. Univ. of Penn. Press.
Claude, A.
1943. *Science,* 97: 451–456.
Cohen, S. S., and E. Chargaff
1940. *Jour. Biol. Chem.,* 136: 243–256.
Cooper. K. W.
1948. *Jour. Exptl. Zool.,* 108: 327–335.
Coulter, C. B.
1921. *Jour. Gen. Physiol.,* 3: 513–521.
Craigie, J., and F. O. Wishart
1934. *Brit. Jour. Exptl. Path.,* 15: 390–398.
Cumley, R. W., and M. R. Irwin
1942. *Genetics,* 27: 177–192, 228–237.
Darlington, C. D.
1937. "Recent Advances in Cytology." J. and A. Churchill, Ltd., London.
1942. *Nature,* 149: 66–69.
1947. *Symposia Soc. Exptl. Biol.,* 1: 252–269.
Daubney, R.
1934. *Jour. Comp. Path.,* 47: 259–295.
Delaporte, B.
1939. *Rev. gén. botan.,* 51: 449–482.
Dodson, E. O.
1948. *Univ. of Calif. Publ. Zool.,* 53 (8): 281–314.
Duran-Reynals, F.
1928. *Jour. Exptl. Med.,* 47: 389–402; 1929, *ibid.,* 50: 327–340.
Duryee, W. R.
1941. "Cytology, Genetics and Evolution," 129–141. Univ. Penn. Press.
Ehrich, W. E., and T. N. Harris
1945. *Science,* 101: 28–31.
Euler, H. von, and E. Brunius
1931. *Z. ImmunForsch.,* 72: 65–92.
Fabergé, A. C.
1942. *Jour. Genetics,* 43: 121–144.

Francis, T.
1947. *Bacteriol. Rev.*, 11: 147–156.
Friedrich-Freksa, H.
1940. *Naturwis.*, 28: 376–379.
Haldane, J. B. S.
1935. *Compt. rend. soc. biol.*, 119: 1481–1496.
Haurowitz, F.
1937. *Klin. Wschr.*, 16: 257–261.
Heidelberger, M.
1946. " Currents in Biochemical Research," 453–460. Interscience, New York.
Heilbrunn, L. V.
1943. " An Outline of General Physiology." W. B. Saunders Co.
Hinton, T.
1946. *Jour. Exptl. Zool.*, 102: 237–252.
Hughes, T. P.
1933. *Jour. Immunol.*, 25: 275–294.
Hydén, H.
1947. *Symp. Quant. Biol.*, 12: 104–114.
Jehle, H.
1948. *Bull. Am. Phys. Soc.*, 23: 39.
Jordan, E. O., and W. Burrows
1942. " Textbook of Bacteriology." W. B. Saunders Co.
Jordan, P.
1940. *Z. ImmunForsch.*, 97: 330–344.
Kabat, E. A.
1939. *Jour. Exptl. Med.*, 69: 103–118.
Koltzoff, N. K.
1928. *Bot. Zeit.*, 48: 345–369.
1938. *Biol. Zhurn.*, 7: 3–46, Moscow.
Kuwada, Y.
1937. *Cytologia,* Fujii Jub. Vol.: 389–402.
1939. *Cytologia,* 10: 213–256.
Landsteiner, K.
1946. " The Specificity of Serological Reactions." Harvard Univ. Press.
Langmuir, I.
1938. *Jour. Chem. Physics,* 6: 873–896.
Libby, R. L., and C. R. Madison
1947. *Jour. Immunol.*, 55: 15–26.
Lipschütz, B.
1925. *Zbl. Bakt.*, 96: 222–227.
London, F.
1943. *Pub. No. 21, A. A. A. S.*, 141–149.
Ludford, R. J.
1928. *Proc. Roy. Soc.*, 102B: 406–418.
Luria, S. E.
1947. *Proc. Nat. Acad. Sci.*, 33: 253–264.
Manouélian, Y.
1912. *Ann. Inst. Pasteur,* 26: 973–985.

Mazia, D.
1941. *Symp. Quant. Biol.,* 9: 40–46.
Mazia, D., and L. Jaeger
1939. *Proc. Nat. Acad. Sci.,* 25: 456–461.
Mazia, D., Hayashi, T., and K. Yudowitch
1947. *Symp. Quant. Biol.,* 12: 122–130.
McClintock, B.
1933. *Z. ZellForsch.,* 19: 191–237.
Mirsky, A. E.
1943. *Advances in Enzymol.,* 3: 1–34.
1947. *Symp. Quant. Biol.,* 12: 143–146.
Mirsky, A. E., and H. Ris
1947. *Jour. Gen. Physiol.,* 31: 7–18.
Mitchell, J. S.
1943. *Brit. Jour. Radiol.,* 16: 339–343.
Mudd, S.
1932. *Jour. Immunol.,* 23: 423–427.
Muller, H. J.
1922. AM. NAT., 56: 32–50.
1941. *Symp. Quant. Biol.,* 9: 290–308.
Muller, H. J., and T. S. Painter
1929. AM. NAT., 63: 193–200.
Northrop, J. H.
1938. *Jour. Gen. Physiol.,* 21: 335–366.
Oster, G.
1947. *Jour. Gen. Physiol,* 31: 89–102.
Painter, T. S.
1934. *Genetics,* 19: 448–469.
1945. *Jour. Exptl. Zool.,* 100: 523–547
Painter, T. S., and A. N. Taylor
1942. *Proc. Nat. Acad. Sci.,* 28: 311–317.
Pauling, L.
1940. *Jour. Am. Chem. Soc.,* 62: 2643–2657.
Pauling, L., and M. Delbrück
1940. *Science,* 92: 77–79.
Perla, D., and J. Mormorston
1941. "Natural Resistance and Clinical Medicine." Little, Brown and Co.
Pollister, A. W., and G. I. Lavin
1944. *Anat. Rec.,* 89: 557–558.
Rich, A. R.
1944. "The Pathogenesis of Tuberculosis." Charles C. Thomas Co.
Ris, H.
1945. *Biol. Bull.,* 89: 242–257.
Rivers, T. M.
1928. *Am. Jour. Path.,* 4: 91–124.
Rothen, A.
1947. *Jour. Biol. Chem.,* 168: 75–97.
1948. *Jour. Am. Chem. Soc.,* 70: 2732–2740.
Rothen, A., and K. Landsteiner
1939. *Science,* 90: 65–66.

Sabin, F. R.
1939. *Jour. Exptl. Med.*, 70: 67–82.
Sakamura, T.
1927. *Protoplasma,* 1: 537–565.
Schmidt, C. L. A.
1944. "The Chemistry of Amino Acids and Proteins." Charles C. Thomas Co.
Schoenheimer, R.
1942. "The Dynamic State of Body Constituents." Harvard Univ. Press.
Schrader, F.
1944. "Mitosis." Columbia Univ. Press.
Schramm, G.
1941. *Ber.*, 74B: 532–536.
Schultz, J.
1941. *Symp. Quant. Biol.*, 9: 55–65.
Schultz, J., Caspersson, T., and L. Aquilonius
1940. *Proc. Nat. Acad. Sci.*, 26: 515–523.
Sevag, M. G.
1946. *Advances in Enzymol.*, 6: 33–127.
Sheffield, F. M. L.
1931. *Ann. Appl. Biol.*, 18: 471–493.
Small, J.
1929. *Protoplasma Monographien*, Vol. II, Berlin.
Spiegelman, S.
1946. *Symp. Quant. Biol.*, 11: 256–277.
Spiegelman, S., and M. D. Kamen
1946. *Science*. 104: 581–584.
1947. *Symp. Quant. Biol.*, 12: 211–223.
Svedberg, T.
1938. *Ind. Eng. Chem.*, 10: 113–129
Svedberg, T., and K. O. Pederson
1940. "The Ultracentrifuge." Clarendon Press, Oxford.
Topley, W. W. C., and G. S. Wilson
1946. "The Principles of Bacteriology and Immunity." Williams and Wilkins Co.
Treffers, H. P.
1944. "The Handbook of Medical Physics," 627–636. Chicago.
Troland, L. T.
1917. AM. NAT., 51: 321–350.
Van Atta, E. W.
1932. AM. NAT., 66: 93–95.
Vlasow, J.
1945 *Jour. Physics, U. S. S. R.*, 9: 25.
White, A., and T. F. Dougherty
1946. *Ann. N. Y. Acad. Sci.*, 46: 859–882.
Wright, S.
1941. *Physiol. Rev.*, 21: 487–527.
1945. AM. NAT., 79: 289–303.
Wrinch, D.
1947. *Science,* 106: 73–76.
Yamaha, G.
1935. *Cytologia,* 6: 523–527.

MISCELLANEA MEGADRILOGICA

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I

"DO EARTHWORMS GROW BY ADDING SEGMENTS?"

THE usual answer, in the past, to the question quoted as title of this note, has been in the affirmative. Production of new segments has even been said to be continuous (Hyman, 1940, p. 522), presumably throughout life. Although all such answers appear to have been expressions of a rather widely held opinion, there is little supporting evidence in the literature.

A negative answer to the question, in recent years, has increasingly been asserted. Indeed, the negative occasionally has been converted into this positive,—just hatched juveniles already possess their full complement of segments (Moment, 1946, p. 492). Authorities, when cited, have been Sun and Pratt (1931).

The raw data of Sun and Pratt were measurements and segment counts of 750 specimens supposedly all of *Helodrilus foetidus* = *Eisenia foetida* (Savigny) 1826. The material was in three samples (of 250 each): (1) just hatched juveniles, (2) immature intermediates between the first and the next groups, and (3) mature (clitellate) worms. Number of segments in those three groups respectively was as follows: 37–136, mean 99.65; 59–125, mean 105.5; 67–124, mean 105.0.

Juveniles having more segments than the adults also had been recorded by Morgan (1895) and in the same species. Number of segments 84–106, average of 97 segments for forty adults, and 67–111, average of 92.5 segments for twelve just hatched juveniles. Morgan passed over that curious situation without comment. Sun and Pratt considered the problem and concluded that individuals with the larger numbers of segments were selectively eliminated before maturity.

Certain casual references to internal anatomy and regeneration indicate a possibility of presence of some other species in Morgan's material. The Sun and Pratt cocoons are now known to have been obtained from an uncontrolled compost heap in which at least four species were present (*vide* II). All of those species fall within the *foetida* size range and criteria for distinguishing their cocoons from each other and those of *foetida* are still unknown. Almost as difficult would have been taxonomic identification of just hatched juveniles. No evidence has been found to indicate that any of the above-mentioned authors attempted specific identification of their cocoons or juveniles or took such precautions as would render that identification unnecessary.

Sun and Pratt recognized a four per cent. contamination by a second species in their adult sample but suggested that difference in breeding habits might have provided taxonomic homogeneity for one or both of the other samples. Very little is known, even today, about life histories of earthworms, and still less as to possible effects thereon of such special habitats as are provided by various sorts of compost heaps. Accordingly it appears to be theoretically possible that percentages of contamination were higher in other series and especially in the intermediate one which was collected at a later date than the other two samples.

At conclusion of the breeding season the clitellum regresses and it may become, in certain conditions, completely unrecognizable in a period as short as one week. Accordingly the supposedly intermediate sample could have contained worms that had previously been sexual and even at the time the clitellate sample was being collected.

Individuals of *E. foetida* have lived in the laboratory, after operation, for more than four years (Korschelt, 1914). Thus it could have been possible that both the intermediate and the mature samples contained worms that were two or even more years old.

The adult sample, according to the data provided (*vide* II), apparently comprised at least four species. Possibility of presence of still other species even more liable to misidentification can not be ruled out in the circumstances. One of the probable and two of the possible contaminating species have greater numbers of segments than *foetida.*

In spite of the fact that Sun and Pratt have been credited with showing that earthworms hatch with the full complement of segments, their own conclusions were more cautiously stated. " From the data presented it seems clear that it is not safe to assume that *H. foetidus* continues to add new segments from the time of emergence from cocoons until sexual maturity is reached. . . . Obviously the problem can not be definitely settled until young worms with a definite number of segments can be grown to maturity and recounted " (Sun and Pratt, 1931, pp. 46–47).

SUMMARY

Taxonomic heterogeneity of the adult sample, with possibilities of less or greater heterogeneity in the other two samples, theoretical possibilities of considerable age heterogeneity in both immature and mature samples, necessitate confirmation of any conclusions, either as to role of segment formation in growth or as to specific norms in *E. foetida,* derived from analysis of the Sun and Pratt statistics.

II

ON THE EARTHWORM FAUNA OF AN OHIO COMPOST HEAP

The heap is that from which Sun and Pratt (1931) secured material for study of segment formation in normal growth (*vide* I) and for determination of species characteristics by statistical analysis.

All the 750 worms measured at first were thought to be *E. foetida.* Apparently during study of the raw data it was found that ten clitellate specimens were of another species called *Helodrilus subrubicundus* = *Dendrobaena s.* (Eisen) 1874. On each of those ten worms the clitellum covered eight segments, xxii–xxix. As the clitellum of *subrubicunda* begins only on xxv or xxvi the worms were misidentified.

Two North American Lumbricid species do have a clitellum on xxii–xxix: *Bimastos tumidus* (Eisen) 1874 and *B. hempeli* Smith 1915. Both have been recorded from Ohio. Both have been reported from similar habitats, under moss and leaves, in decaying leaves, under bark of decaying logs, in rotten wood. Either species could have been present if the compost heap was mainly or wholly composed of decaying leaves. Balance of probability seems in favor of *hempeli*, if the two forms are really specifically distinct.

In the rest of the clitellate sample (240 specimens) number of clitellar and preclitellar segments was 4–10 and 23–28, respectively. In *E. foetida*, according to the data otherwise available, number of segments is 6–9 but six and nine both uncommon, and 25–26, only rarely 24, possibly very rarely 23 but requiring confirmation. Are there species with a clitellum of 4, 5, or 10 segments, having 23, 27, or 28 preclitellar segments, that might have been confused with *E. foetida?*

A four-segment clitellum is known, in American species of the family Lumbricidae, only in the genus *Eiseniella*. All American varieties have, at most 22, usually fewer preclitellar segments. Furthermore all are limicolous and therefore unlikely to have been present in an ordinary compost heap. A five-segment clitellum may be present in *Dendrobaena octaedra* (Savigny) 1826 and *D. rubida* (Savigny) 1826. A ten-segment clitellum may be present in *Bimastos longicinctus* Smith and Gittins 1915, *Eisenia hortensis* (Michaelsen) 1890, and *Allolobophora chlorotica* (Savigny) 1826. In those species, number of preclitellar segments may be 23 or 24 in *longicinctus* and *hortensis*, 27 or 28 in *octaedra* and *chlorotica*.

Each of those species may have a red color as marked as that presumably responsible for early failure to distinguish *hempeli*. All species fall within the *foetida* size range (to

130 × 5 mm.). All have been recorded from Ohio, excepting *rubida* only. All, with exception of *longicincta* and *hortensis*, have now been reported from compost heaps (*vide* III).

However, the problem is further complicated by the fact that still other species have a similar red color, are known both from compost heaps and from Ohio, and fall within the size range of *foetida.* Two such species, *Eisenia rosea* (Savigny) 1826 and *D. subrubicunda,* have the clitellum on the same segments as *foetida.*

Segment number of all above-mentioned species falls within the *foetida* range (to 125), with exception of *longicincta, chlorotica* and *rosea* in which known maxima are 130, 135 and 150, respectively.

SUMMARY

The data of Sun and Pratt indicate that four species, at least, were present in the adult sample. Balance of probability among various recognizable possibilities favors *B. hempeli, A. chlorotica, D. octaedra.* Presence in the sample, of species with a clitellum on the same segments as in *foetida,* would not have been indicated by the data. Yet those species appear to be even more liable to such misidentification.

III

EARTHWORM FAUNAE OF COMPOST HEAPS

Compost heaps, if of manure, may contain only one species of earthworm, *E. foetida.* As a result, and perhaps also because of common reference to *foetida,* as "*the* manure worm," there has been a rather general tendency to assume that almost any manure pile will contain *foetida,* and that all worms, at least of red color, in such piles are *foetida.* To check on such assumptions and to provide information with regard to compost heaps as uncontrolled sources of cocoons, the data presented below were accumulated.

In each of five manure piles (4 cow and 1 horse) in Hancock County and three (cow) in Cumberland County, Me., the following species were found: *Dendrobaena rubida,*[1] *D. subrubicunda,*[1] *D. octaedra, Allolobophora caliginosa, Lumbricus terrestris.* In four Hancock County and three Cumberland County manure piles (cow and horse) no earthworms were found, although piles appeared to be of about the same age and condition as those with worms. In one pile only (cow), in Cumberland County, was *E. foetida* present and abundant. In that pile no other species was present.

In a pile of rotting leaves in Cambridge, Mass., the following species were found: *A. caliginosa, E. foetida, D. subrubicunda, Bimastos tenuis, Lumbricus castaneus* and *L. terrestris.*

In a pile composed of turf, grass, hay, garbage, etc., in Windsor County, Vt., the following species were found: *A. caliginosa,*[1] *Eisenia rosea,*[1] *D. octaedra,*[1] and *L. terrestris.*[1] In four large manure piles (cow) in the same locality no earthworms were found.

From a Fairfax County, Va., manure pile (cow) several specimens of *A. chlorotica*[1] were secured, in addition to a number of *E. foetida.* The preserved specimens of *chlorotica* show no indication whatever of a supposedly characteristic green color but instead have a red pigment.

In manure brought from Lincoln County, Me., in which only *E. foetida* was found, it was possible, with some care, to separate the cocoons into two groups according to size. Cocoons failed to hatch, but the larger ones almost certainly are not of *E. foetida* and possibly are of *A. caliginosa.* Cocoons obtained from Hancock and Cumberland heaps in June likewise were separable into two groups, one possibly of *caliginosa,* the other presumably of *Dendrobaena* sp. The latter doubtless would have been very difficult to distinguish from those of *foetida* if in the same medium. Whether large *foetida* cocoons are distinguishable from small *caliginosa* cocoons remains to be determined.

The author's thanks are extended to Professor John Welsh for the Lincoln County manure and to Mrs. E. D. Oliver for collecting the Fairfax County worms.

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[1] Not hitherto recorded from the state.

SUMMARY

Seven species of earthworms, found in compost heaps: *D. rubida, subrubicunda* and *octaedra; D. octaedra, A. caliginosa, E. rosea, L. terrestris; A. chlorotica;* are recorded for the first time from the states of Maine, Vermont and Virginia, respectively.

LITERATURE CITED

Hyman, L. H.
1940. AM. NAT., 74: 513–527.
Korschelt, E.
1914. *Zool. Anz.*, 43: 537–555.
Moment, G. B.
1946. *Jour. Exp. Zool.*, 103: 487–506.
Morgan, T. H.
1895. *Quart. Jour. Mic. Sci.*, 37: 395–476.
Sun, K. H., and Pratt, K. C.
1931. AM. NAT., 65: 31–48.

IV

NEGLECTED FACTORS

The problem of whether earthworms form new segments after hatching has been studied hitherto by analysis of statistics of number of segments and measurements of length. To obtain evidence more directly, examination of posterior ends of all available material was made a matter of routine practice for several years.

In every one of a considerable number of species some individuals had no externally recognizable metameric rudiments such as would indicate presence of a growth zone. All such worms, in many of the species, were characterized as follows:

The terminal segment was as large as those preceding and in colored species had pigment about as dense as on other metameres. Setae were approximately the same in size, number and position as in those segments in front. If nephropores were recognizable elsewhere they were also visible on the terminal segment. The anal aperture usually seemed larger and with more irregular margins than ordinarily. Occasionally an anus was abnormal or even absent.

Each worm was always shorter to much shorter than the maximum length of the species and the number of segments was always smaller to very much smaller than the specific maximum. Yet body thickness and segment length, along the whole axis, were definitely greater than in other worms of the same length, occasionally the same as in the very largest mature specimens.

Each of such worms undoubtedly had undergone amputation of a posterior portion of its body. In the majority of cases appearance was such as to suggest that cut margins of gut and body wall had united directly (enteroparietal healing), so that a normally setigerous segment had become anal. Assumption of that new role apparently produced no externally recognizable changes in the metamere in a great majority of the worms. In some individuals of several species of *Eutyphoeus* the terminal segment had lost some setae and size after amputation. More rarely one or two segments in front had also decreased in size.

In forty specimens of *Perionyx simlaensis* (Michaelsen) 1907, posterolateral margins of a terminal, setigerous segment apparently turned mesially to form a vertically slit-like anus on the posterior face of the worm. Separation of those margins showed only a concave depression, the floor of which was mainly white. More or less centrally in that white area, in some worms, was a very small circular opening into the gut.

The white tissue clearly was a cicatrix formed across the posterior surface after amputation (cicatricial healing). The small cicatricial perforation presumably represented an early stage in formation of an anus. In other species a later stage of cicatricial sculpturing showed a vertically slit-like anus and cicatrix reduced to a narrow, ribbon-like perianal band. Laboratory observations have showed that during a period of such cicatricial sculpturing and for some time afterward there was no growth. Later, in some species, and in certain conditions, the perianal band slowly grew into a tail regenerate.

If the cicatrix remained imperforate a different sort of development may follow, at least in the laboratory. Setae fall out of the cicatrized segment which grows smaller until it is reduced, occasionally with one or two metameres next in front, to microscopic rudiments on the posterior face of the worm.

Postamputation developments accordingly may be either of a progressive or regressive nature. Recognition of the latter suggested explanations as to two classes of doubtful specimens frequently encountered.

In one of those classes worms differed from unregenerate amputees described above mainly as follows: Size of the last two to four or five segments decreased posteriorly and irregularly, not gradually as in normal specimens. Setae usually were wholly or partly lacking and when present often appeared to be large for size of segment. In perichaetine species setae were scattered or in one or more patches. Anus always present. These worms apparently can be regarded as unregenerate amputees unevenly affected in a limited number of posterior segments by regressive reorganization.

In a second class regression may be responsible for the condition of the anterior three to five segments in a terminal region of five to eight segments, but other developments seem necessary to explain condition of the last two or three segments. Laboratory studies (on other species) indicated that there might be involved one or more of the following processes: dedifferentiation, reorganization of two or three segments from one (or perhaps two) dedifferentiated segments, very slow regeneration of one to three segments. As in the previous class, amputation apparently preceded whatever developments were responsible for the unusual conditions.

Percentages of unregenerate amputations in a few series collected during war years (much data of a similar nature lost in Rangoon) are shown in Table I. Percentages ob-

TABLE I

PERCENTAGE OF UNREGENERATE POSTERIOR AMPUTATIONS

Species	Locality	Condition	Number of specimens	Number of specimens with amputation	Percentage of amputation	Group
Eutyphoeus incommodus	Rae Bareli	clitellate	210	68	80.0%	1
" "	Lahkaoti	clitellate	90	4	4.4%	1
" *nicholsoni*	Allahabad	clitellate	60	10	16.6%	1
" *waltoni*	"	clitellate	110	9	8.1%	1
Lampito mauritii	Robertsganj	juvenile to clitellate	884	6	.7%	2
" "	Sohagi	juvenile to clitellate	616	18	2.9%	2
Perionyx sansibaricus	Allahabad (October)	adult	4,126	146	3.5%	2
" *simlaensis*	Saharanpur	large juvenile	477	40	8.3%	2
Eisenia foetida	Darjiling	juvenile to clitellate	711	28	3.9%	2
Allolobophora caliginosa	Simla	adult ...	179	9	5.0%	2
Pheretima hawayana	Gangtok	clitellate	42	7	16.6%	3
" *diffringens*	Darjiling	juvenile to clitellate	145	27	18.6%	3
" *robusta*	Darjiling	juvenile to clitellate	42	6	14.2%	3

tained when tail regeneration is also included as evidence of amputation are shown in Table II. All cases of the two classes considered just above were omitted from both tables.

Amputation may take place at any time after hatching. In 11 juveniles of *Perionyx sansibaricus* Michaelsen 1891, bred in the laboratory, three lost posterior portions early in the first week after hatching. Specimens of *Lampito mauritii* Kinberg 1867 about one mm. thick that had already undergone amputation were found and some of them already had caudal regenerates. Specimens of *Eisenia foetida* (Savigny) 1826 two mm. thick or slightly smaller, with well-developed tail regenerates were collected.

In Table II it has been possible to present some of the data in age groups determined according to size, or the state of development of certain reproductive structures. These groupings show a general tendency for percentage of amputation to increase with age. That tendency is well marked in a series of *L. mauritii* (Robertsganj) in which juveniles were quite arbitrarily separated into two groups according to size. In *P. simlaensis* a further grouping of adults

TABLE II

PERCENTAGE OF POSTERIOR AMPUTATION (UNREGENERATE AND REGENERATE)

Species	Locality	Number of specimens	Unregenerate	Regenerate	Totals	Percentage of amputation
L. mauritii	Sohagi	286–205–125	5–7–6	54–61–39	59–68–45	20.6–33.1–36%
" "	Robertsganj	214–162–326 132	0–2–3–1	34–44–97–55	34–46–100–56	15.8–28.3–30 6–42 4%
P. sansibaricus		4,126	146	371	517	12.5%
E. foetida		241–470	8–20	25–92	33–112	14.1–23.8%
A. caliginosa		0–111 68	0–4–5	0–22–3	0–26–8	0–23.4–11.7%
P. hawayana		0–0–22	0–0–7	0–0–2	0–0–9	0–0–40.9%
P. diffringens		1–19–125	0–2–25	1–0–8	1–2–33	100–10.5–26.4%
P. robusta		0–11–31	0–1–5	0–0–3	0–1–8	0–9.0–25.8%

Explanation. One figure only, mostly clitellate but with a few large juveniles. Set of two figures: juveniles and aclitellates-clitellates, set of three figures: juveniles-aclitellates-clitellates. Set of four figures: small juveniles-large juveniles-aclitellates-clitellates.

Anterior amputation. In the series included in table above there were anterior amputations, evidence of amputation (regeneration), or indication of regenerated amputation (homoeosis) as follows.—*L. mauritii*, one unregenerate aclitellate after amputation at 10/11. *P. sansibaricus*: 95 head regenerates, of which specimens 23 had a tail regenerate and 5 had an unregenerate posterior amputation. (Homoeosis not determined.) *P. simlaensis*: 18 head regenerates, four specimens also with tail regenerates. *E. foetida*: one unregenerate anterior amputation, one anterior regenerate, three homoeotics. *P. hawayana*: one anterior regenerate. *P. diffringens*: two, unregenerate, following amputation at 13/14 and some level behind 18/19.

In case of unregenerate anterior amputation healing was cicatricial. The cicatrix was imperforate and without other indication of sculpturing.

according to clitellar characteristics was possible. The percentages are as follows:

Small juveniles	31	per cent. amputees
Large juveniles	28.7	per cent.
Presexual aclitellates	36.3	per cent.
Clitellates	50	per cent.
Postsexual aclitellates	100	per cent.

Although total percentages of amputation by species may seem high even greater have been found. In a collection secured from axils of plantain leaves at Darjiling every specimen—*E. foetida, Pheretima diffringens* (Baird) 1869—had undergone a posterior amputation. (No anterior amputations.)

If all specimens of the two special classes considered above are in reality amputees, as they do appear to be, then in a number of Himâlayan collections one hundred per cent. of the material of three species of *Pheretima* had undergone amputation.

The higher percentages in Table I for species of *Eutyphoeus* seem to be associated with inability to regenerate posteriorly. Each of the specimens in that table was examined with special care but without finding any indication of regeneration. Nor were tail regenerates found in course of identification of more than 9,000 specimens during a three-year survey of fauna of a region centering at Allahabad (Gates, 1945). Still larger numbers of specimens had been identified during a twenty-year survey of the Burmese fauna but without finding tail regenerates. In many Burmese and Indian species of the genus a regenerate should have been easily recognizable because of the dark pigmentation of unregenerate regions. Contrary then to the general belief (Hyman, 1940, p. 513) some species of earthworms appear to be unable to regenerate posteriorly. Among such species the common *Lumbricus terrestris* L. apparently must be included.

In reports on determinations of segment number in such earthworm species as *E. foetida* (Morgan, 1895; Sun and

Pratt, 1931; Moment, 1946; etc.) and *L. terrestris* (Pearl and Fuller, 1905), there is nothing to indicate that natural amputation and its effect on segment number were taken into consideration. Some of the skewness in the graphs of segment number (Pearl and Fuller, Sun and Pratt) doubtless was due to presence of amputees.

In fact it now seems quite probable that those specimens of *L. terrestris* in the third to fifth length class which puzzled Pearl and Fuller because of the relatively small number of segments were amputees. Instead then of being younger and still growing slowly they may well have been among the oldest in the sample and quite unable to grow because of inability to regenerate. Frequency of amputation may have been responsible for failure to find in the Ann Arbor sample individuals approximating maximal records of body length and segment number for the species.

Except for omission of any mention of the matter in previous studies it would appear to be unnecessary to emphasize the fact that a worm lacking a portion of its main axis is just as abnormal as a lizard without its tail or a man without his legs. Lest percentages of amputations recorded above from Oriental samples be attributed to some local peculiarity of no general importance it may be here noted that not only in India are earthworms attacked by mammals, birds, reptiles, amphibia, arthropods and molluscs. In each recently-examined collection of American material amputees (regenerate and unregenerate) have been present.

Amputations may also be due to an internal enemy. Keilin (1925, p. 171) pointed out that brown bodies containing gregarine cysts and nematode larvae accumulate in the coelomic cavities of the posteriormost segments as a result of which a posterior portion of the body is broken off. As his term "parasitic autotomy" may be thought to have implications of direct action by parasites on hosts it should be noted that the autotomy appears to be a reaction to the mechanical pressure of the accumulations. In coelomic accumulations at the posterior end of Oriental earthworms the writer has found masses of unconsolidated gregarine cysts, spores, nematode ova, as well as the brown bodies which occasionally contained immobilized dipterous larvae. Similar accumulations, giving the last few segments a characteristic appearance, have been observed frequently in American earthworms, most recently in those from the compost heaps mentioned in III. Some evidence is available to indicate that autotomy may result from direct action of certain dipterous larvae. Autotomy, independent of parasites, is also possible as some who have tried to keep earthworms in the laboratory have discovered to their consternation.

SUMMARY

Posterior amputation may occur naturally in all postembryonic stages of the life history of earthworms. Frequency increases with age, sometimes reaching one hundred per cent. After amputation healing may be enteroparietal or cicatricial. In the latter case subsequent developments may be either progressive or regressive. Some species apparently are unable to regenerate posteriorly.

LITERATURE CITED

Gates, G. E.
1945 *Proc. Nat. Acad. Sci. India,* 15: 44–56.
Hyman, L. H.
1940. Am. Nat., 74: 513–527.
Moment, G. B.
1946. *Jour. Exp. Zool.,* 103: 487–506.
Morgan, T. H.
1895. *Q. J. M. S.,* 37: 395–476.
Pearl, R., and Fuller. W. H.
1905. *Biometrika,* 4: 213–229.
Sun, K H., and Pratt, K. C.
1931. Am. Nat., 65: 31–48.

DIFFERENTIAL PRECOCITY AT MEIOSIS IN DROSOPHILA

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1. Introduction

Drosophila miranda Dobz. is peculiar in respect of its chromosome set in that the female has 10, while the male has only 9 chromosomes. Three of the nine have been identified by Dobzhansky (1935) as the sex chromosomes. While two of the largest chromosomes with submedian centromeres represent the usual XY complex, the third sex chromosome, designated X″, is much smaller and, like the autosomes, it has a terminal—or almost terminal—centromere (Plate I, 1). Thus the sex-determining mechanism of the male *D. miranda* consists of three chromosomes, namely the X′, X″ and Y. The extensive breeding tests carried out by Dobzhansky (1935) have shown that only two kinds of sperms are effective: those with X′X″, and those with Y. The behavior of the three sex chromosomes proved to be of a unique character. This had been expected, for in other species while the sex chromosomes form reciprocal chiasmata, the autosomes pair by quadruple chromatid attraction (Darlington, 1936).

Chromosome structure and behavior in another strain of *D. miranda* (called Whitney) was investigated by the author (Koller, 1939) (Plate I, 2). This strain was derived from flies collected by Professor Th. Dobzhansky near the locality of Mount Whitney, California, while the first strain was collected in the state of Oregon on the Olympic Mountains. Owing to the lack of information concerning the influence of environmental factors on meiotic chromosome behavior in *miranda*, the cytological basis of X′X″-Y segre-

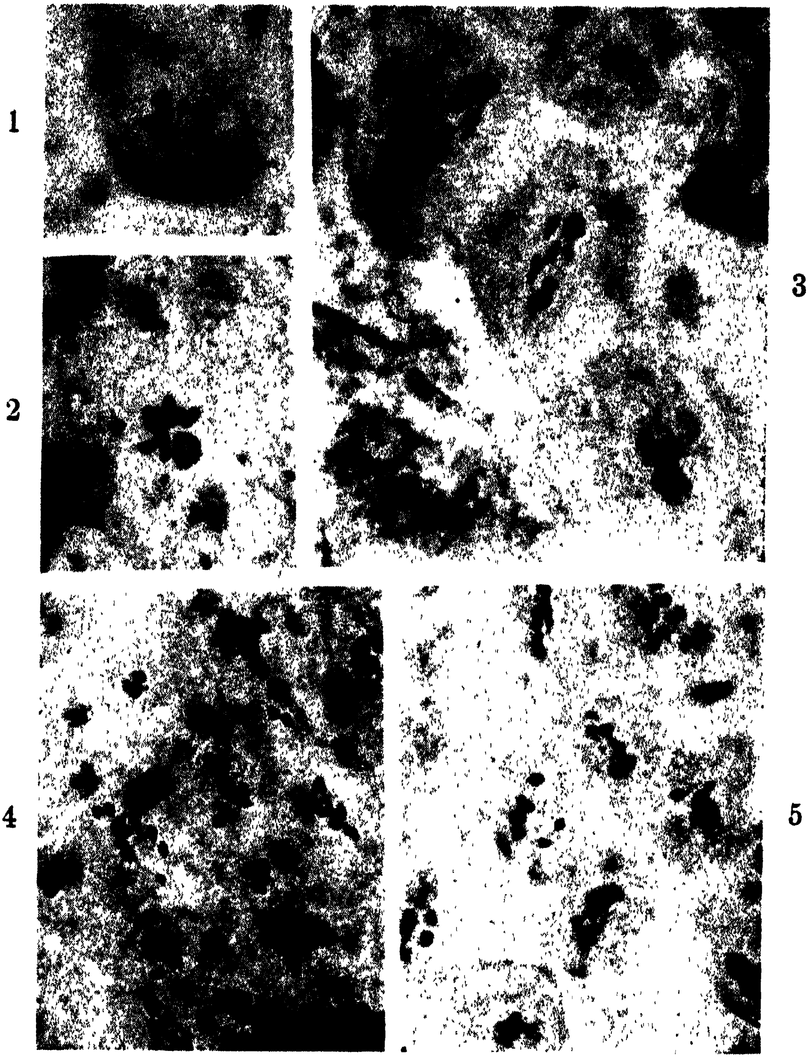

PLATE I

1. Mitotic chromosomes of male *Drosophila miranda,* Olympic strain. The two large chromosomes with sub-median centromeres represent the X′ and Y. X″ is one of the rod-shaped chromosomes. Magnification × 1500.

2. Mitotic chromosomes of male *D. miranda,* Whitney strain. The small dot chromosomes, representing the third autosomal pair, are not visible. (Figs. 1 and 2, squash preparation from ganglia, stained with aceticlacmoid.) Magnification × 1500.

gation was not understood clearly at the time when Dobzhansky (1935) and Koller (1939) described the new species.

During studies of spermatogenesis in the Whitney strain and in the crosses between Whitney and Olympic, however, I had found indications that temperature, and perhaps the age of the flies, may influence the association of chromosomes at meiosis, which is indeed highly variable. Further investigations were therefore carried out and some of the results were demonstrated during the Seventh International Congress of Genetics at Edinburgh in August, 1939. Owing to the outbreak of the second World War, however, which followed the closing of the Congress, the publication of the results was postponed until such time as the author considered opportune. The data which will be presented below were obtained while the author was a member of the staff of the Institute of Animal Genetics, University of Edinburgh.

2. TEMPERATURE AND CHROMOSOME BEHAVIOR

During spermatogenesis of *D. miranda* bred at 24° C., there is always a high number of spermatocytes in metaphase I with three free sex chromosomes; the stage of meiosis having been identified by the behavior of the two large autosomal chromosome pairs. The presence of free sex chromosomes and associated autosomes in the same cell can be due, either to lack of pairing between the sex chromosomes during the preceding prophase, or to a separation of the associated sex chromosomes in advance of the autosomes. At the time of the first investigation (1939), the

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3. Anaphase I of meiosis from pupae bred at 16° C., showing the synchronous segregation of sex chromosomes and autosomes. (Section stained with Feulgen's method and counterstained with acetic carmine.) Magnification × 1500.

4. Spermatocytes from pupae bred at 24° C., showing the lack of synchronization between the behavior of sex chromosomes and autosomes. Magnification × 1300.

5. Spermatocytes from 5-day old fly bred at 16° C., showing precocious separation of the sex chromosomes. (Figs. 3–5 are sections stained with Feulgen's method and counterstained with acetic carmine.) Magnification × 1300.

former possibility was considered to be responsible for the high frequency of spermatocytes with " free " sex chromosomes. Configurations were seen however, some of which were illustrated (*cf.* Koller, 1939, Figs. 20, 24, 26, 28, 31, 32, 34, 35), suggesting that the sex chromosomes associate and form a trivalent during meiosis.

Chromosome behavior of *D. miranda* bred at 24° and 16° C. was compared and the effect of temperature is summarized in Tables 1 and 2. In a variable proportion of spermatocytes, the chromosome configuration could not be analyzed and scored separately. It is very probable that many of these configurations were trivalents with chromosomes so closely associated as to be indistinguishable. The data show that the number of spermatocytes with a recognizable sex trivalent (Fig. 1) is much greater, and with three " unpaired " or " free " sex chromosomes (Figs. 8, 9) much smaller, at 16° C. than at 24° C. The orientation of

TABLE 1

CHROMOSOME CONFIGURATION OF X′X″Y COMPLEX IN 100 SPERMATOCYTES AT 24° C (2-DAY PUPAE)

Strain	No. of spermatocytes not analyzed	No. of spermatocytes with sex trivalent (Fig. 1)	No. of spermatocytes with sex bivalent and univalent		No. of spermatocytes with sex chromosome univalents (Fig. 8)	Total no. of spermatocytes
			$\widehat{X'Y}$-X″ (Fig. 3)	$\widehat{X''Y}$-X′ (Fig. 5)		
Olympic	17	37	22	8	16 (3) *	100
Whitney	8	39	38	5	20 (7)	100
O♀ × W♂	21	12	33	7	27 (1)	100
W♀ × O♂	27	29	27		17	100
Percentage	18.3	26.8	30.0	5.0	20.0	...

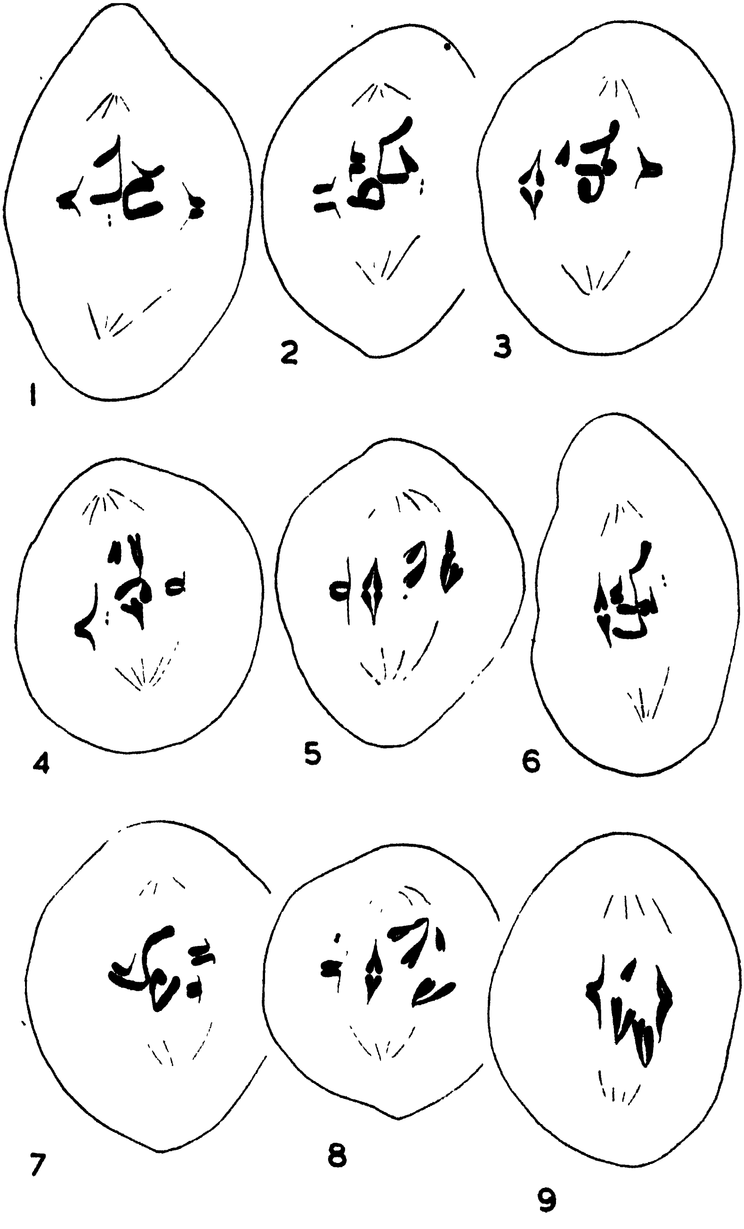
* No. of spermatocytes with all sex chromosomes mis-oriented.

TABLE 2

CHROMOSOME CONFIGURATION OF X′X″Y COMPLEX IN 100 SPERMATOCYTES AT 16° C (2-DAY PUPAE)

Strain	No. of spermatocytes not analyzed	No. of spermatocytes with sex trivalent	No. of spermatocytes with sex bivalent and univalent		No. of spermatocytes with sex chromosome univalents	Total no. of spermatocytes
			$\widehat{X'Y}$-X″	$\widehat{X''Y}$-X′		
Olympic	16	53	27	4		100
Whitney	24	40	18	15	3	100
O♀ × W♂	19	36	25	14	6 (2) *	100
W♀ × O♂	26	39	17	10	6	100
Percentage	21.2	42.0	21.7	10.7	3.7	

* No. of spermatocytes with all sex chromosomes mis-oriented.



FIGS. 1–9. Camera lucida drawings of spermatocytes of *Drosophila miranda*, showing the various configurations of the sex chromosomes X′, X″ and Y during meiosis (Figs. 1–5 at 16° C., Figs. 6–9 at 24° C.). (Squash preparations stained with orcein.) Magnification × 1500.

the "free" sex chromosomes has shown a quantitatively normal 2–1 distribution. This fact strongly suggests that they were paired previously. Out of the 1,200 spermatocytes analyzed, only 2.4 per cent. had mis-oriented free X′, X″ and Y (combined data of Tables 1, 2 and 3). In these cells the sex chromosomes no doubt had failed to pair during meiotic prophase. It seems, therefore, that the three sex chromosomes pair during meiotic prophase and metaphase, but at high temperatures, the precocious anaphase noted by Darlington (1934) to be a property of the sex chromosomes (which undergo a true meiosis) is exaggerated, thus obscuring their pre-metaphase relationship. When flies are bred at 16° C., however, the duration of association of the sex chromosomes is almost as long as that of the autosomes, *i.e.*, the onset of anaphase stage is simultaneous for both types (Plate I, 3).

TABLE 3

CHROMOSOME CONFIGURATION OF X′X″Y COMPLEX IN 100 SPERMATOCYTES OF PUPAE (P) AND ADULT FLIES (A) BRED AT 16° C

Strain	Age	No. of spermatocytes not analyzed	No. of spermatocytes with sex trivalent	No. of spermatocytes with sex bivalent and univalent		No. of spermatocytes with sex chromosome univalents	Total no. of spermatocytes	No. of individuals
				$\widehat{X'Y}$–X″	$\widehat{X''Y}$–X′			
Olympic	Pupae	21	43	24	10	2	100	7
Whitney	Pupae	25	58	10	2	5	100	4
Percentage	. .	23.0	50.5	17.0	6.0	3.5		
Olympic	Adult	17	24	30	17	12 (2) *	100	22
Whitney	Adult	19	36	18	13	14 (4)	100	18
Percentage	.	18.0	30.0	24.0	15.0	13.0		

* No. of spermatocytes with all three sex chromosomes mis-oriented.

It was observed that irrespective of temperature differences, the X″ separates more frequently from the trivalent configuration than either of the larger sex chromosomes (Figs. 2, 3, 4). The class of spermatocytes in which one of the large sex chromosomes (presumably X′) was free while the other was associated with the small X″ (Fig. 5) is scored separately in the tables.

3. AGE AND CHROMOSOME BEHAVIOR

Slight individual variations in the frequencies of different chromosome configurations were noticed previously. The number of flies in each age group, however, was too small to show significant differences (*cf.* Koller, 1939, Table IV).

In the present study the age groups were more clearly defined by selecting 2-day-old pupae and 5-day-old adult males bred at 24° C. and 16° C. Many more adult flies were of course required to obtain the necessary 100 spermatocytes for analysis than pupae. Table 3 contains the data obtained from pupae and adult flies bred at 16° C. only; the data of 24° C. having been lost during the war. The proportion of spermatocytes with one or all three sex chromosomes unpaired was smaller in pupae than in adult flies (26.5 per cent. and 52.0 per cent., respectively) (Plate I, 4, 5).

The comparison of data suggests that the precocity of sex chromosomes during meiosis is more exaggerated in adult flies than in pupae, *i.e.*, the duration of chromosome association is reduced in the former. The influence of age is discernible in spite of the effect of low temperature, which tends to reduce the precocity of the sex chromosomes.

4. THE METHOD OF PAIRING OF THE SEX CHROMOSOMES

The data presented above leave no doubt that the three sex chromosomes of *D. miranda* are associated during meiotic prophase and metaphase and form a trivalent, as was postulated by Darlington (1936).[1] My data suggest that the duration of this association varies with temperature and age. The shape of the trivalent and breeding tests together indicate that the two X's separately pair with the Y and separately disjoin from it, leading to the formation of Y and X′X″ gametes only. It seems that the configuration of the two large sex chromosomes of *D. miranda* is very similar to that described by Darlington (1934) in *Drosophila pseudoobscura.* He showed that the X and Y chromosomes are

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[1] Cytological evidence for this was provided by Cooper (*Genetics*, 1946: 31: 181–194).

paired by two reciprocal chiasmata near the centromere, a view since supported by genetic evidence (Mather, 1943). From this similarity it may be inferred that the metaphase association of X'Y in *D. miranda* is similarly brought about by two reciprocal chiasmata. In all the 436 sex trivalents analyzed the same arm of the Y pairs with X' and with X"; the latter is associated near the distal end. It seems, therefore, that X' and Y have one single pairing segment; in this respect, *D. miranda* differs from *D. pseudoobscura* in which the X and Y chromosomes have two pairing segments, one in each arm. Only two spermatocytes were seen in which the free arm of Y might have been paired with X". In both instances, which may be considered to be exceptional, the X" was attached to the Y chromosome at the distal end. But whether terminal or intercalary the association never resembled a chiasma, it always resembled the parallel pairing of the autosomes. There are two facts which can be reconciled with this interpretation: (a) trivalent configurations were seen in which the X" showed "reversed" pairing, the X" and Y pairing segments being associated in an inverted sense (Fig. 7); (b) the frequency of precocious separation of the X" from the trivalent is much higher than that of the X' chromosomes. If the association between X" and Y were of the same type as that between X' and Y, *i.e.*, by true chiasmata, the incidence of "free" X' and X" should be the same, which is not the case. It can be assumed, therefore, that the X"-Y association is of a different nature from that existing between X' and Y. It resembles that of the autosomes, but the pairing region being extremely short—hence the apparent inversion and hence, also, the premature separation.

It is concluded, therefore, that the Y chromosome of *D. miranda* has two kinds of pairing properties during meiosis: it pairs by two reciprocal chiasmata with X' and by quadruple chromatid attraction with X" (Fig. 10). Translocation between autosome and sex chromosome in *Drosophila* should give the combination with both types of pairing.

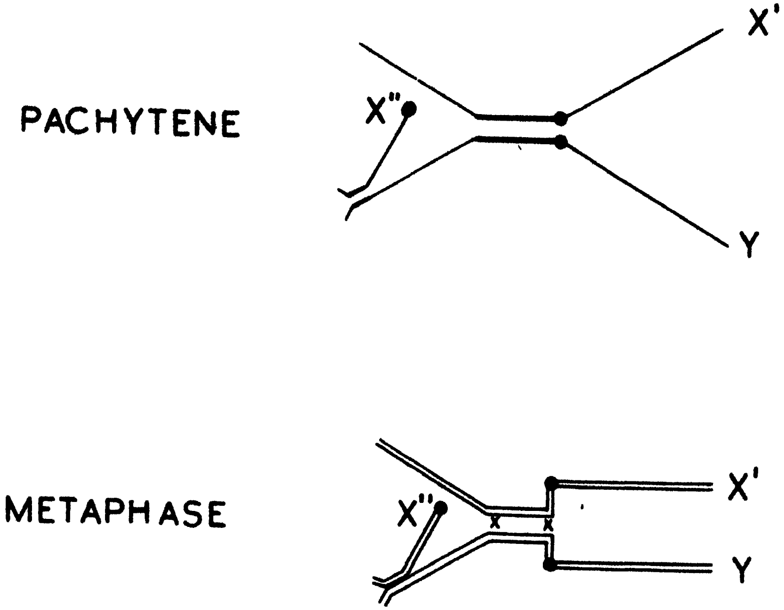

FIG. 10. Diagram showing the two kinds of association between the three sex chromosomes of *D. miranda*.

The analysis of the salivary gland nuclei in *D. miranda* shows that the Y chromosome has several euchromatic segments, the structure of which closely resembles that of the third autosomal chromosome (MacKnight, 1939). One of these particular segments could have retained the characteristic pairing property of the autosomes, which would thus provide the physical basis of the association between X″ and Y.

5. THE PHYSICAL BASIS OF DIFFERENTIAL CHROMOSOME BEHAVIOR

The experimental data presented above indicate that when flies are bred at high temperatures, the sex chromosomes display a pronounced precocity, shown by shortening of the duration of metaphase association of these chromosomes as compared with that of the autosomes. At low temperatures, however, the precocity of the X′X″Y complex is greatly reduced, as shown by the fact that these chromosomes remain associated for a longer period during metaphase of meiosis. On the other hand, there is no evidence that temperature changes affect the duration of metaphase association of the autosomal chromosome pairs; it seems from the data that the temperature effects are restricted to the sex chromosomes only. It is of some interest to note that at 24° C. the proportion of spermatocytes with "free" X′ is less than at 16° C., while that of those with "free" X″ at 24° C. is higher than at 16° C. The X″ apparently reacts to temperature changes more like the autosomes than the large X′ and Y sex chromosomes. Because the autosomes do not respond in the same way or to the same degree as the sex chromosomes during meiosis to temperature changes, it may be assumed that they differ in chemical organization, and that the mechanism of association of the autosomes and of the sex chromosomes is conditioned by this difference.

A comparison of meiotic behavior of the sex and autosomal chromosomes indicates that the rate of nucleic acid charge, consequently the rate of chromosome synthesis itself, is greater in the sex chromosomes than in the autosomes. It is shown by precocious condensation of the former during prophase of meiosis. This phenomenon, known as "pycnosis," is common in the heterogametic sex of a great number of animal species (*cf.* White, 1945). The experimental data, given above, thus indicate that in *D. miranda* the rate of nucleic acid charge of the sex chromosomes is affected by temperature and perhaps by age also. In the golden hamster it was demonstrated that besides age, diet and breeding season are other factors, which can influence the nucleic acid charge in a particular segment of the Y chromosome (Koller, 1945). This is another proof that the reaction of sex chromosomes is greater to environmental changes than that of the autosomes. Though the ultimate cause which determines the rate of nucleic acid charge of a particular chromosome is not known, we assume on the evidence presented that the different nucleic acid cycle forms the physical basis of the differential chromosome behavior in the heterozygous sex of *D. miranda.*

6. SUMMARY

1. The three sex chromosomes—X′, X″ and Y—of male *Drosophila miranda* Dobz. pair, and under suitable conditions, form a triple figure during meiosis.
2. The association of X′ and Y, which have a pairing segment near the centromere, is by two reciprocal chiasmata.
3. The association of X″ and Y is assumed on indirect evidence to be by an extreme degree of somatic pairing, similar to that found between autosomes.
4. Change in temperature affects the relative timing of anaphase for the two types of pairing. The duration of meiotic pairing of the sex chromosomes is longer in flies bred at 16° C. than in those bred at 24° C. It is also longer in larvae or pupae than in adult flies. The behavior of autosomes is independent of temperature and age.
5. The difference in reaction of sex chromosomes and autosomes to environmental factors suggests a difference in their organization. It seems that they have a different rate of nucleic acid cycle during meiotic prophase, which perhaps conditions their characteristic pairing properties.

LITERATURE CITED

Darlington, C. D.
1934. *Genetics,* 19: 95–118
1936. AM. NAT., 70: 74–75.
Dobzhansky, Th.
1935. *Genetics,* 20: 377–391.
Koller, P. C.
1939. *Jour. Genet.,* 38: 477–492.
1946 *Proc. Roy. Soc. B,* 133: 313–326.
MacKnight, R. H.
1939. *Genetics,* 24: 180–201.
Mather, K.
1943. *Biol. Reviews,* 18: 32.
White, M. J. D.
1945. "Animal cytology and evolution." Cambridge University Press. Pp. 375.

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CONFERENCE ON PROBLEMS OF GENERAL AND CELLULAR PHYSIOLOGY RELATING TO FERTILIZATION. I[1]

ACTIVATION OF EGGS, FERTILIZATION AND EARLY DEVELOPMENT AS AFFECTED BY ULTRAVIOLET RAYS

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ULTRAVIOLET radiations, like many other agents, initiate artificial parthenogenesis in eggs. They also have other profound effects on eggs even when they do not initiate development. While sunlight may occasionally produce such effects in nature, it is unlikely, since the actinic radiations of sunlight are not very intense and penetrate only if the water is clear. Neither do such radiations enter more than a few millimeters into tissues, therefore gonads are safe from them. This is in striking contrast to the ionizing radiations such as X-rays which may penetrate tissues deeply and selectively destroy germinative or "embryonic" tissues such as the germinative epithelium, the blood-forming tissues and the stratum germinativum of the skin (see Giese, 1947, for references).

The studies which have been made with ultraviolet rays are therefore comparable to studies made with the effects of poisons, narcotics and anesthetics which do not occur naturally but which interfere with the normal course of development. The aim of such research is to gain an insight into the nature of fertilization and early development by interfering with the various processes concerned. If the data from a number of lines of such investigation are brought together, some features of fertilization and development are clarified.

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I shall first discuss the initiation of development of eggs by ultraviolet light, then the effect of these rays on gametes and early development following fertilization.

Jacques Loeb (1914) was the first to initiate development in eggs with ultraviolet light. Using *Arbacia* and *Chaetopterus* and exposing them to the entire radiations of a mercury lamp in quartz, he observed that membranes were formed and that some of the eggs so activated went on to develop to the two- and the four-celled stage. Even if oxygen were eliminated by bubbling hydrogen through the suspension of eggs, the latter were activated. He found that a high temperature was deleterious, room temperature leading to disintegration, but that a low temperature such as 12° C. was satisfactory. When such activated eggs were put into hypotonic sea water they developed into larvae. Chaetopterus gave rise to larvae without cell division.

Lillie and Baskerville (1922*a* and *b*) studied the activating effect of these rays on starfish eggs. They were particularly interested in what they called partial activation, that is, the exposure to light decreased the time necessary for other agents such as heat or butyric acid to act. Heat of 33° C. was especially effective after ultraviolet, but was ineffective if applied before the irradiation.

Tschakhotine (1935*a* and *b*) was able to activate *Pholas* and sea urchin eggs by what he called ultraviolet puncture since he irradiated with a spot only 5 μ in diameter. The local spot irradiated shows either a bleb, or a depression, or one followed by the other. What interests us here is that the membrane first formed at the point of irradiation extends itself over the entire egg. He did not study the speed of formation, but this would be interesting since the action is so localized and in this respect more or less comparable to sperm puncture. Some eggs are activated by ultraviolet light of λ 2537 Å (Giese, 1939) others not (Giese, 1938*a*).

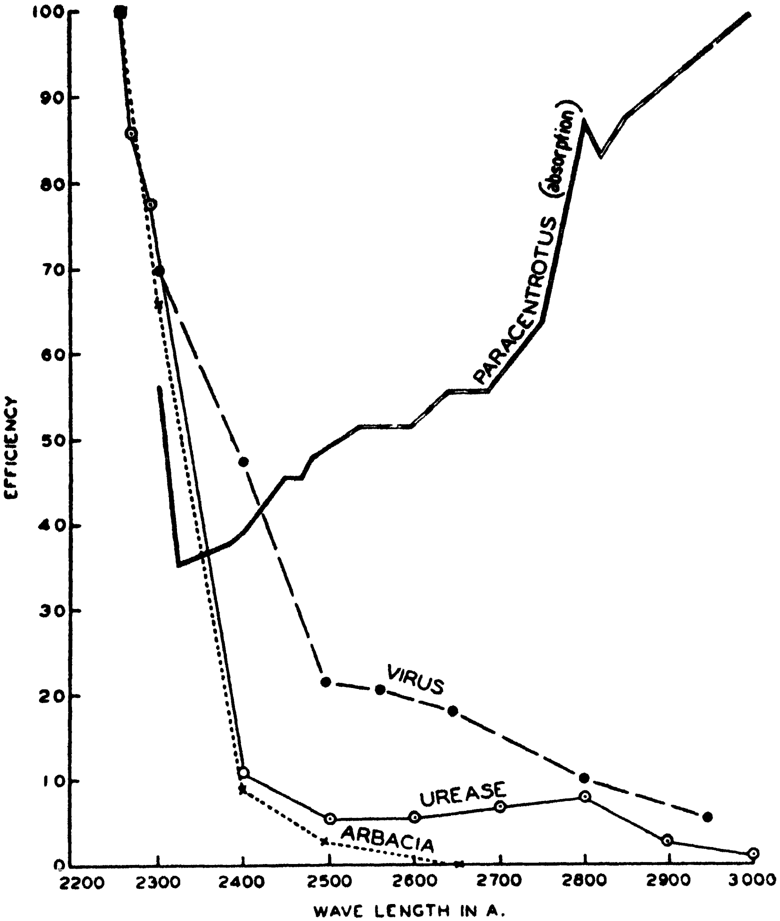

FIG. 1. Action spectrum for 50 per cent. activation of Arbacia eggs compared to destruction of urease and of virus and absorption by Paracentrotus eggs (from Hollaender, 1938).

Loeb (1914) surmised that only the short wavelengths of ultraviolet light produced activation, since insertion of a thin glass coverslip which cuts off wavelengths shorter than

3000 Å between the eggs and the source of light prevented activation of sea urchin eggs. Hollaender (1938) made a study on *Arbacia punctulata* at Woods Hole, using known dosages of monochromatic light. He found that large dosages of light at 2804 Å gave very little activation, and even at 2654 Å the effect was still only slight, but at shorter wavelengths the effects increased with decrease in wavelength. Activation is greater the shorter the wavelength over the span used. It is probable that even eggs reported unresponsive to ultraviolet rays would be susceptible if exposed to shorter wavelengths, but no one has yet made such a comparative study.

In Fig. 1 is shown what is known as an action spectrum, since it compares the action of different wavelengths of the spectrum on a particular biological reaction, in this case, activation. The action spectrum is thought to correspond fairly closely to the absorption spectrum of the substance being affected by the light. Such a deduction, it will be remembered, enabled Warburg to identify the nature of indophenol oxidase. However, such a deduction can be made only if the action spectrum has a characteristic structure by which it can be identified with the absorption spectrum of some material. As seen from Fig. 2 this activation action spectrum does not correspond to the absorption spectra of the nucleoproteins and the non-conjugated proteins which are the two commonest constituents of the cell. It is therefore necessary to attempt to match the action spectrum with the absorption spectrum of some of the other constituents of the cell: proteins lacking aromatic amino acids, lipids, carbohydrates, salts, and water. The latter three lack specific absorption (Caspersson, 1936). Both the lipids and the proteins lacking aromatic amino acids (such as the histones and protamines) have only a sort of general absorption in the short ultraviolet, increasing with decrease in wavelength (Caspersson, 1936). Both lipids and proteins occur in the membrane and Parpart and Dziemian have shown lipids and proteins in the ratio of 1:1.7. The protein in the stroma of the erythrocyte has been analyzed

(Beach, Erikson, Bernstein, Williams and Macy, 1939) and shown to contain both tyrosine and tryptophane. Such a protein would therefore show specific absorption. By the process of elimination it would appear that the action spectrum of activation corresponds most closely to the absorption by membrane lipids, but the evidence is not conclusive. The action spectrum of another effect of ultraviolet light, hemolysis, which is thought to be an effect on the cell surface, resembles that of activation in that action is very slight even at λ 2804 Å, rising rapidly below 2530 Å (Sonne, 1929). This similarity makes more probable the supposition that both hemolysis and activation result from action on constituents of the membrane. One other surface effect suggests itself for study in comparison to activation and hemolysis—permeability. However, studies made by Reed

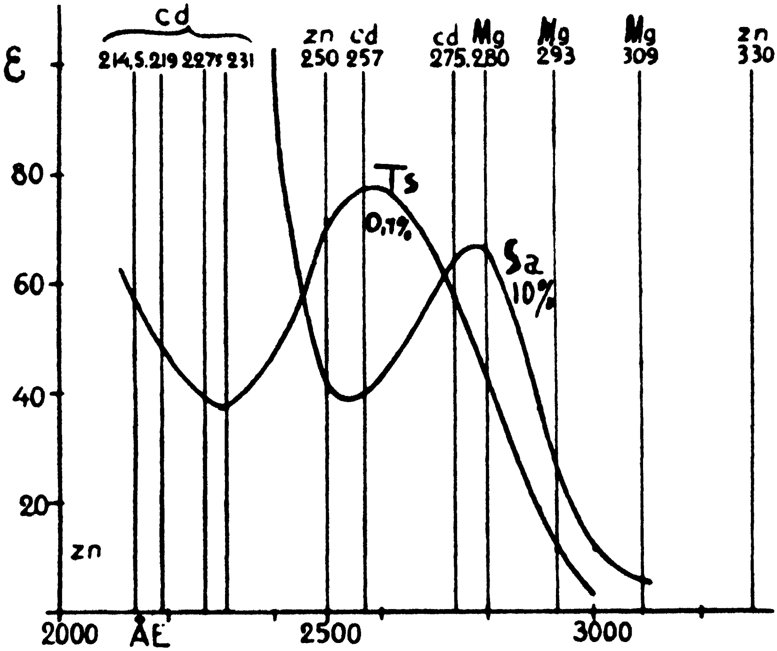

FIG. 2. Comparison of absorption by thymusnucleic acid and serum albumin Note difference in absorption maxima as well as differences in relative absorption (from Caspersson, 1936).

(1948) indicate no change in permeability of sea urchin eggs unless the cell is damaged, therefore no action spectrum could be determined.

Harvey and Hollaender (1937; 1938) have studied the activating effects of various monochromatic wavelengths of ultraviolet light on eggs stratified by centrifugation (10,000 × gravity) and on the fragments of such eggs centrifuged to the breaking point. They showed that the white halves are more sensitive to the rays than whole eggs. The red halves containing pigment are sensitive to all wavelengths from 2650–3050 Å. This indicates that not only the surface but also the pigment present deeper in the cell is active in initiating development. This series of experiments also confirms the already generally accepted view that activation is rather non-specific, and that the agents which bring it about set off some sort of trigger mechanism. However, the extent to which development follows such activation is dependent on the stimulus. Another conclusion which may be drawn from these experiments is that since the white halves only have the female pronucleus and since both halves may be activated by radiations, the nucleus is not necessary for activation.

That more than just the surface of the cells is affected by these radiations is shown by many cortical changes and by the alterations in the production of jelly and in the formation of the fertilization membrane after raying. Just (1933) believes that injury resulting from ultraviolet raying is largely cortical as judged by the changes in the jelly secretion in *Nereis* following raying. Spikes (1944) working with *Lytechinus* and Reed (1944) with *Strongylocentrotus* have described unilateral effects of ultraviolet in suppressing membrane formation on the irradiated side. Spikes reports that the cleavage plane passes through the axis of irradiation. Reed and Whitaker (1941) showed that polarized or unilateral plasmolysis occurs in *Fucus* treated unilaterally with ultraviolet light. Their interpretation is that the cytoplasm below the membrane is involved, being somewhat gelated by action of the rays. Their results suggest that parts of the cell other than the membrane are affected by the radiations.

Much more clear-cut evidence for a general effect of ultraviolet light on eggs is indicated in the work in which the eggs are irradiated and then fertilized with unirradiated sperm, or *vice versa.* An example of the immediate effects of radiation on the early cleavages of eggs is shown in Fig. 3. The retardation increases with increase in dosage, but the increase is not linear, successive increments causing progressively lesser effects. Two questions arise: (1) Is the division of the egg merely retarded or (2) is development qualitatively altered? Data are available which show that if the embryos are examined at the blastula, gastrula or pluteus stages one finds that there is a progressive retardation but that the embryos so formed are to all appearances entirely normal, unless the dosage is excessive. In the latter case the blastulae are filled with cells of irregular size and may never differentiate (Giese, 1938*a* and *b*). One may say that small amounts of ultraviolet radiations merely

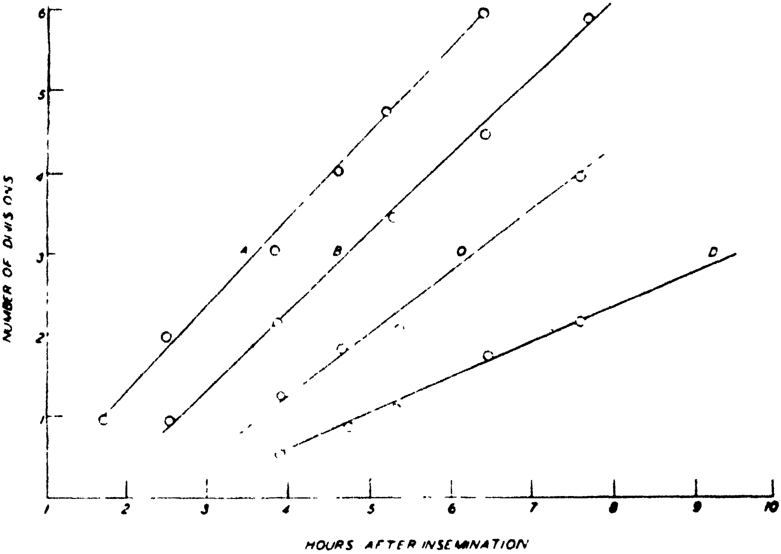

FIG. 3 Effect of λ 2804 Å on rate of division of eggs of the sea urchin, *Strongylocentrotus purpuratus* (from Giese, 1938*b*).

retard the divisions but excessive amounts interfere with normal development. Perhaps it is necessary to emphasize a point here which does not come out in the figures, namely, that the threshold for retardation is very high. Thus, from absorption data it is possible to show that the egg may absorb as much as 10^9 or 10^{10} quanta without showing any retardation in division even for short wavelengths. Only when ten times this amount has been absorbed are definite signs of retardation observed. If we assume for purposes of calculation that the egg contains only protein molecules of the size of albumin then the egg would have something of the order of 10^{12} molecules. Even if we are wrong by a factor of 10 or more in this calculation the amount of radiations which an egg can absorb without being affected is impressive. The fact that the larvae developing from retarded eggs not treated with excessive dosages still develop to all appearances normally indicates the remarkable recovery of irradiated eggs (Giese, 1938*a* and *b*).

Not all eggs respond in the same manner. Thus the eggs of Echinoderms show only retardation following smaller dosages and irregularities only after larger dosages. In the eggs of the mosaic type tested, for example, *Nereis*, *Urechis*, and *Chaetopterus* (Giese, 1946) there is a tendency for abnormalities to appear even after relatively small dosages. Another effect observed with the latter type of egg is that in some cases, *e.g.*, *Nereis*, one may observe a localized effect on one side of the unilaterally irradiated egg and later on the embryo.

The action spectrum for retardation of division by irradiation with ultraviolet light has been worked out in the case of *Strongylocentrotus purpuratus* (Giese, 1946). As seen in Fig. 4 the action spectrum for retardation of the irradiated egg is a fair match for the absorption spectrum of albumin or globulin or similar proteins (Fig. 2). The maximum effect appears where such proteins have a maximum, namely, 2800 Å. This suggests that these proteins are affected and their transformation results in the retardation of division. The action spectrum demonstrates clearly that the substance affected which leads to the retardation of division is different from that which is affected in activation and artificial parthenogenesis. The egg is therefore affected by ultraviolet light in more than one locus in the cell and in more than one way. Although this action spectrum does not implicate the nucleus or the nucleoproteins, there is evidence that the nucleus is affected. Studies by Stevens (1909) and later by Schleip (1923) on *Ascaris* eggs show that when whole eggs are irradiated a wide range of chromosomal abnormalities follow: thus clumping, excessive chromatin diminution, lagging of chromosomes and thickening of the membranes were observed among other things. Even if the nucleus was screened and only the cytoplasm was irradiated, various abnormalities appeared similar to those observed when the entire egg was irradiated. However, larger dosages were required. This suggests that cytoplasmic injury may lead to formation of toxic materials that affect the chromosomes which have not been rayed.

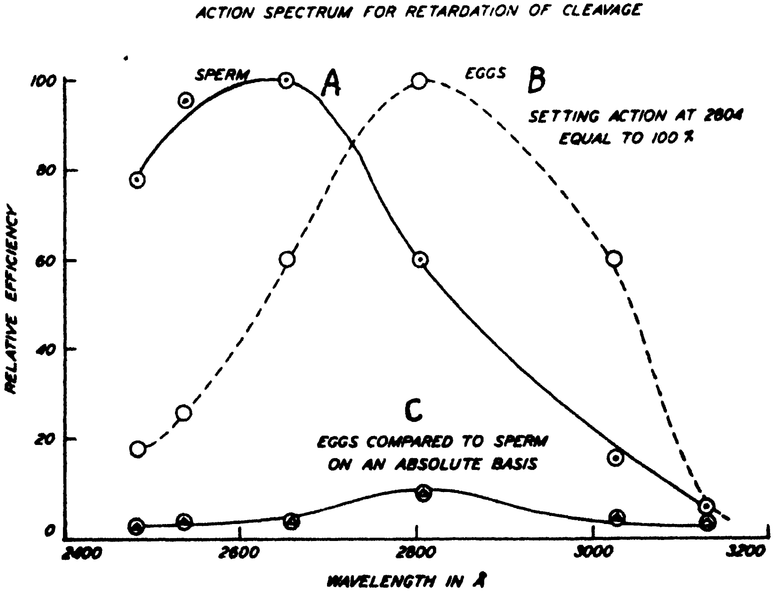

FIG. 4. Action spectra for sperm and eggs of *Strongylocentrotus purpuratus* (from Giese, 1946)

Sperm directly rayed are much more susceptible than eggs. This is easily demonstrated by inseminating untreated eggs with irradiated sperm and studying the delay in cell division. A graded series is obtained with dosage. If the effects on eggs and sperm are compared on the basis of the light actually absorbed by each, then only one millionth the energy at λ 2654 Å is required to produce effects on the sperm as on the egg. This indicates a sensitivity of an entirely different order of magnitude. If the effectiveness on sperm of different wavelengths is compared, an action spectrum is obtained which corresponds more closely to the absorption spectrum of nucleoproteins (Giese, 1946) than to any other known cellular constituent. This is not surprising in view of the fact that between 60 to 80 per cent. of the contents of the sperm are nucleoproteins. The sperm nucleus is quickly protected once it penetrates the egg and the susceptibility of fertilized eggs is similar to the unfertilized.

Perhaps of all the experiments on retardation of division of eggs the most striking are those on suppression of division of eggs by irradiation. Suppression of cleavage is possible within the first thirty minutes after insemination. After this, larger and larger dosages are necessary. Just before division even very large dosages are ineffective; however, cleavage is followed by cytolysis. The cell is set to divide and even excessive damage no longer prevents it.

Just what synthetic reactions in the cell are affected by ultraviolet radiations is not clear, but an inkling has been gained from studies involving ultraviolet photography and Feulgen staining. Caspersson and his coworkers have shown that desoxyribose nucleic acid is synthesized during its mitotic cycle and the desoxyribose nucleic acid rises during the prophases and reaches its highest concentration during the metaphase. It falls during the anaphase and reaches its minimum concentration during the interphase. The nucleolus contains ribosenucleic acid and perhaps is the center of formation of this compound. It disappears in many cases during the formation of the chromosomes and reappears before interphase. Parallel with the nucleic acid cycle is the cycle of complex and simple proteins. When the nucleoproteins of the desoxyribose type are at a maximum, the complex proteins containing the aromatic groups (which can be studied spectrophotometrically) are at a minimum and the histones (without aromatic nuclei) are at a maximum.

This cyclic change, the exact significance of which is not at present clear, is interfered with by radiations. At least it has been noted in a number of instances that injured or dying cells absorb ultraviolet light more strongly (Luyet and Gehenio, 1936), and Brumberg and Larionow (1946) claim that one can not see the nucleus as distinct from the cytoplasm in photographs taken at all wavelengths between 2540–2750 except after prolonged irradiation. They claim that the radiations have injured the cells so that the photographs show dying or dead cell constituents. Irradiation of cells may lead to an accumulation of ultraviolet absorbing materials, as shown by Loofbourow (1948). Loofbourow presents evidence which indicates that cells deeply injured by ultraviolet radiations lose, through their membranes, not only nucleotides but also vitamin B factors and amino acids, which filtered free of cells stimulate growth and respiration in other unirradiated cells. Spectrophotometric analyses show that the nucleotides which appear are present in proportions other than would result from a mere breakdown of the nucleoproteins of the cells. This suggests synthesis of certain of these nucleotides by the injured cells. It is possible that the darkening of the injured cells is due to an accumulation of nucleotides since ultraviolet light does not discriminate between the nucleotides and nucleic acids, the absorption being largely dependent on the quantity of purine or pyrimidine bases present in the compounds.

It should be possible to study some of the chemical changes which occur in unfertilized eggs activated by ultraviolet by the photographic and spectrophotometric method. Harvey and Lavin (1944) have made a study of the unfertilized and the fertilized Arbacia egg up to just before cleavage. They show that nucleoproteins are present throughout the cytoplasm and that they are concentrated in the nucleolus and the chromatin at the germinal vesicle stage. As the germinal vesicle breaks down the nucleoprotein increases in the nucleus, but the amount in the cytoplasm does not change much. Perhaps a slightly greater concentration appears about the nuclear membrane which is thought by some to be the seat of formation of these materials. The cyclic change in constituents after irradiation has not been studied in any case.

The following general conclusions may be drawn from the account presented:

(1) The mature egg ready for fertilization seems to need only a trigger action of some change to set off activation. Ultraviolet light may act as the trigger but more nearly normal development is obtained if the radiation is followed by a secondary treatment such as heat or hypertonic sea water.

(2) The ultraviolet action spectrum suggests that the material affected by these rays is probably in the membrane and is either lipid or protein devoid of aromatic amino acids, or both. It is probably not an effect on nucleoproteins or on the common unconjugated proteins.

(3) The effect of ultraviolet radiations is deleterious and never beneficial to both sperm and eggs, sperm being more sensitive than eggs.

(4) So far as suppression of cleavage is concerned the egg is more sensitive in the early stages than later. It appears that once certain processes have been set in motion no amount of radiation short of complete cytolysis will stop them.

(5) The threshold dosage which an egg can tolerate before it is injured is very high. A fair proportion of the protein molecules will have absorbed some energy before any signs of retardation show up.

(6) Some evidence indicates that ultraviolet light interferes with the manufacture or conversion of nucleoprotein. Absorption studies suggest that nucleoproteins or nucleo-

tides accumulate in the irradiated cell which becomes more opaque to these radiations.

(7) There is as yet no evidence for parthenogenesis produced by use of ultraviolet treated sperm; if sperm are introduced, no matter how battered they are, they always seem to combine with the egg nuclei and at least influence the division (Giese, 1946).

(8) Ultraviolet light, like so many other interfering agents, is not a specific tool for stopping a particular process in the cell. General injury to the cells is indicated by the fact that different action spectra are obtained for activation and retardation of division.

(9) While ultraviolet light used as a single technique may not be so promising for further studies on fertilization and development, it may be that small dosages followed by other agencies such as heat (Lillie and Baskerville, 1922*a*, *b*) might be a fruitful field of study. The reversal of ultraviolet effects by visible radiation concurrently applied or subsequently applied may also be interesting (Whitaker, 1942) and has recently been studied on viruses and bacteria (Kelner, 1949).

LITERATURE CITED

Beach, E. R., Bn N. Erikson, S. S. Bernstein, H. H. Williams and I. G. Macy
1939. *Jour. Biol. Chem.*, 128: 339.
Brumberg, E. M., and L. T. Larionow
1946. *Nature*, 158: 663–664.
Caspersson, T.
1936. *Skand. Archiv. f. Physiol.*, Suppl. No. 8 to Vol. 73: 1–151.
De Robertis, E. D. P., W. W. Nowinski and F. A. Saez
1948. "General Cytology." Ch 6, 119–133.
Giese, A. C.
1938*a*. *Biol. Bull.*, 74: 330–341.
1938*b*. *Biol. Bull.*, 75: 258–265
1939. *Physiol. Zool*, 12: 319–328.
1945. *Physiol. Zool.*, 18: 223–250.
1946. *Biol. Bull.*, 91: 81-87.
1947. *Quart. Rev. Biol.*, 22: 253–282.
Harvey, E. B., and A. Hollaender
1937 *Biol. Bull.*, 73: 365
1938. *Biol. Bull.*, 75: 258–265.

Harvey, E. B., and G. Lavin
1944. *Biol. Bull.*, **86**: 163–168.
Hollaender, A.
1938. *Biol. Bull.*, 75: 248–257.
Just, E. E.
1933. *Archiv. Entwicklungs.*, 130: 495–516.
Kelner, A.
1949. Proc. Natl Acad. Sci., 35: 73–79.
Lillie, R. S., and M. L. Baskerville
1922*a*. *Am. J. Physiol.*, 61: 57–71.
1922*b*. *Am. J. Physiol.*. 61: 272–288.
Loeb, J.
1914. *Science*, 40: 680–681.
Loofbourow, J. R.
1948. *Growth Symposium*, 12: 75–149.
Luyet, B., and P. M. Gehenio.
1936. *Biodynamica*, No. 11: 1–8.
Parpart, A. K., and A. Dziemian
1940. Cold Spring Harbor Symp. Quant. Biol, 8: 17– .
Reed, E. A.
1944. *Anat. Rec.*, 87: 39.
1948. *Jour. Cell. Comp. Physiol.*, 31: 261–280.
Reed, E. A., and D. M. Whitaker
1941. *Jour. Cell. Comp. Physiol.*, 18: 329–338
Schliep, W.
1923. *Arch. Zellforschung*, 17: 289–367.
Sonne, C.
1929. *Arch. Physical Therapy, X-rays, Radium*, 10: 239–252
Spikes, J.
1944. *Jour. Exp. Zool.*, 95: 89–102.
Stevens, N. M.
1909. *Arch. Entwicklungsmech.*, 27: 622–639
Tschakhotine, S.
1935*a*. *Compt. Rend. Soc. Biol.*, 119: 1394.
1935*b*. *Compt. Rend. Soc. Biol.*, 120: 714.

DISCUSSION

CHAIRMAN ENGLE: Thank you, Dr. Giese. The work was not only carefully done and presented, but you did it in exactly thirty minutes. It is now open for discussion.

DR. WILLIAM E. BERG: Is it possible to determine the degree of absorption by the cytoplasm at a particular wavelength? What would be the absorption by the first millimeter of cytoplasm of the egg?

DR. GIESE: Such measurements have been tried on sea urchin eggs by Vles. The egg being a lens makes a difficult system to study. However, approximations may be obtained in the following way: We take a suspension of eggs and determine the amount of light of a given wavelength passing through, but there are considerable scattering errors. For this we can correct by measuring visible "absorption," which is scattering since the egg is colorless, and extrapolating into the ultraviolet. At wavelength 2537 Å practically all the light is taken up by an egg so that one egg thickness is sufficient to absorb all incident light. Longer wavelengths are absorbed less completely—the absorption being about 76 per cent. at 2804 Å. Absorption falls off rapidly to about 25 per cent. in the visible. This correction might be applied to the ultraviolet, multiplying by a factor obtained from the equation for scattering of light by particles. Approximately half the ultraviolet light wave would appear to be scattered.

Dr. A. M. Schechtman: I would like to ask whether you have tested the effect of various dosages on eggs after cleavage has started. In the literature in embryology one finds quite a number of references to stimulation by low doses of ultraviolet rays. I wonder whether after cleavage had begun, one might notice a stimulatory effect rather than only an inhibitory effect.

Dr. Giese: The only clear-cut case of stimulation I can remember of eggs is that described by Chase on several species of eggs studied at the Hopkins Marine Station. Irradiation was performed before fertilization, the polar bodies appeared a little early, and the first cleavage was slightly stimulated, and I don't really know whether it is truly significant. I don't know of any other careful studies in which any significant stimulation was observed.

Dr. Schechtman: Would you consider it a safe statement to say that in normally activated eggs or eggs activated by artificial means, on the whole, ultraviolet treatment, in any dosage, would have a retarding or an injurious effect?

Dr. Giese: In general I think you can make that statement. I have tabulated all cases in the literature where stimulation was reported. The best analyzed is that of Loofbourow (see *Growth Symposium,* 12: 75–149, 1948). In that case the stimulation results from growth factors, members of the Vitamin B series, amino acids and nucleotides which leak out of the ultraviolet injured cells. The filtrates have been analyzed chemically and duplicated with artificial sources containing the same concentrations with the same stimulating effect.

DR. SCHECHTMAN: In the forms with which you have worked there is relatively no growth in the stages that are involved whereas working with the chick or tissue cultures one has both a growth process and differentiation which might give us two types of effects with which to deal.

DR. GIESE: In Loofbourow's laboratory a study was made with tissue culture, but it proved too complicated to work with and most of the work has been done with yeast cells. However, Loofbourow's associates did get the same sort of stimulatory effects with chick tissue culture. There is also one other good case of great stimulation reported for Stichicoccus but it is a genetic effect. In all other cases reported for the protozoa, inadequately controlled cultures were used so that I don't think any conclusion is justified.

DR. TYLER: Following the report of the reversal of the lethal effect of ultraviolet radiation by visible light in bacteria we have performed similar experiments with sea urchin sperm. We have run five sets of experiments. The first four were increasingly positive. The fifth one was quite negative. At present we are quite undecided about the ability of visible light to reverse the effect of ultraviolet on the sea urchin sperm.

DR. LINUS PAULING (California Institute of Technology): I think it would be interesting if you were getting effects that depended on only a few quanta. Since the sperm is so much smaller than the egg, and the efficiency of irradiating sperm is ten times as great, it would appear that, on the basis of energy per unit area, your figure of 10^9 for an egg becomes 10^2 or 10^3 for a sperm.

DR. GIESE: If one determines the energy absorbed by the sperm, again with these rough corrections I mentioned, it turns out that the sperm is a million times more sensitive than the egg.

DR. PAULING: Aren't the observations good enough so one can determine the number of quanta involved from the fluctuation theory?

DR. GIESE: How accurate would they have to be? These scattering correction factors introduce a good deal of uncertainty.

DR. PAULING: You would observe the effect for an individual fertilized egg once in a while after very small amounts of irradiation, and then with increasing frequency with larger amounts. In these fluctuations, if the effect is clean-cut, you don't need to average data. You look at each egg and see what has happened.

DR. GIESE: We are always working with populations of eggs of 200 or so. Since they divide synchronously, and since the threshold is so high, I rather doubt that you can find any effects after a small dosage. If the sperms are irradiated with a small dosage, no effect on eggs fertilized with such sperm can be seen.

DR. PAULING: I think there is still a possibility of observing the effect of a small number of quanta if the normal behavior is uniform enough to permit you to recognize the abnormalities individually.

DR. DELBRUCK: The crucial point is, can you determine the fraction of effect or non-effect on sperm to the following categories: less than one per cent., between ten and ninety per cent., to within one-tenth of one hundred per cent.?

DR. GIESE: I haven't made any attempt of that sort. I think it could be done.

CHAIRMAN ENGLE: Those are very important points raised, particularly this whole business of relative absorption of the sperm and egg, one having a high percentage of nucleoprotein and the other being high in ordinary proteins.

DR. DANIEL PEASE: A problem which has interested me considerably is the mechanism of sperm penetration through the egg membrane. There is plenty of reason, of course, to believe that the gel of the egg cortex has a good deal to do with the penetration. In view of the radiation effects upon cytoplasm it would seem to me pertinent to study the effect of radiation on the actual formation and development of the fertilization cone. It may be particularly sensitive to radiation effects. I suppose it could be done in an egg which has a cone which persists for maybe fifteen minutes or so. I don't know of any such experiments in the literature, though, do you?

DR. GIESE: I don't know of any study of that sort. Usually the eggs fertilized perfectly well, though the sperm or the egg had been irradiated.

DR. PEASE: I suppose it is quite possible that you would need massive doses to produce a block.

DR. GIESE: The ultraviolet treated sperm always seems to participate in the formation of a zygotic nucleus as far as we know. This is in contrast to the sharp effect Packard got after ionizing radiations. In that case a sperm given a large enough dose would still enter and activate the egg, but it would then become inactive.

DR. EDWARD L. CHAMBERS: I am interested to know if radiation produces a reversible gelation of the surface of the egg. This might be studied with unilateral radiation.

DR. GIESE: Dr Spikes has done such experiments and I shall refer your question to him.

DR. JOHN D. SPIKES: I don't know whether there is much evidence for reversal. Apparently it only takes small amounts of radiation to have some sort of gelling effect.

DR. SPIKES: Is there a difference in the effects of heat and visible light following ultraviolet exposure of eggs or sperm?

DR. GIESE: I think heat and visible light would have different effects. Experiments with heat indicate that even extremely small amounts of radiation sensitize the protoplasm. For example, Paramecium irradiated with a small dosage at 2480 Å is unaffected in any detectable way. The animals go on dividing as if nothing had happened. Yet with a dosage of heat which does not injure controls the animals are killed. Comparing different wavelengths, one obtains an action spectrum which resembles absorption by serum albumin. Dr. Pauling has suggested that perhaps the molecules of enzymes affected by ultraviolet light which would remain oriented and go on functioning even after they absorb the light, on application of higher temperatures, become disoriented with respect to each other and death results. Visible light, on the other hand, is thought to reverse ultraviolet damage, as Dr. Tyler mentioned.

DR. ERWIN HAAS: I also believe that light and heat would have quite different effects, especially since it is the visible light which seems to be active in reversing the destructive action of ultraviolet irradiation. If it could be shown that blue light was most effective one might suspect that growth factors are produced here by photochemical decomposition of yellow enzymes which exhibit their maximum light absorption in the blue region of the spectrum. The formation of a photo derivative of the prosthetic group of yellow enzymes by irradiation with visible light of low intensity has been discovered by Warburg and Christian and has been determined more quantitatively in 1935 by Theorell.

CHAIRMAN ENGLE: One of the advantages of this kind of a meeting is that we can have all day to discuss these things, and many of the discussions are most pertinent when you button-hole the speaker in the corridor and get him to tell what he thinks about it. I think we should keep reasonably to the schedule and proceed to the next presentation by Edward Novitski, California Institute of Technology, on "Action of X-rays at Low Temperatures on the Gametes of Drosophila."

ACTION OF X-RAYS AT LOW TEMPERATURES ON THE GAMETES OF DROSOPHILA[1]

DR. EDWARD NOVITSKI

California Institute of Technology

PROBABLY more is known about the effects of irradiation on Drosophila than in any other organism. Here the effects usually studied are not those which come under the general heading of physiological effects but rather those of gene and chromosomal mutation. Undoubtedly there are some basic similarities of these two superficially different kinds of effects; the subject of this presentation is one such similarity, the relation of the X-ray induced mutation rate to one such physiological state, temperature.

The work with Drosophila has yielded a reasonable thermodynamic concept of the process of mutation. According to this theory, the energy of the secondary electrons produced by the X-rays, which is far in excess of the energy required to break the most stable chemical bond, may dissipate itself eventually as a chemical change in the gene itself, and that change may be detectable as a gene mutation. As a corollary to this, it is obvious that the temperature of the medium surrounding the gene and the chromosomes at the time of irradiation should have little if any effect because the increment of thermal energy in those limits where work with living material is feasible is negligible in comparison with the energy released by absorption of the radiation. However, if a temperature effect were to exist one might suppose for simple thermodynamic reasons that genes would mutate somewhat more readily at high than at low temperatures.

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[1] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.

The existence of an effect of temperature during irradiation has been tested for by Drosophila workers several times, and the results have been conflicting. Oddly enough, the positive results which have been reported have been in just the opposite direction to that expected; that is, the effectiveness of the irradiation appeared to increase at the lower temperature. Since this conflicts with the above notion concerning the mutation process, additional data have been collected bearing on the point.

The class of mutation studied here is that of sex-linked lethals in Drosophila, which is susceptible to an objective quantitative analysis. Without going into description of the mechanical procedure of analyzing for this type of mutation, it may be mentioned briefly that these lethal mutations kill the animal carrying them if a normal compensating allele is not present.

The first phase of the work involved the study of the effects of very low temperatures applied during irradiation, compared with the effects of the same X-ray dose at higher temperatures. For this study, it was found possible to treat Drosophila at temperature levels as low as −5° C. It was found that the mutation rate per roentgen unit did increase at the lower temperatures, the magnitude of the increase being about 75 per cent. Without presenting the raw data here, it may be stated simply that the differences between the two sets were such that they would have occurred as random variations in a single normally distributed population in only once out of five hundred trials. Similar highly significant differences apply to the results described below.

It was found that the mutation rate could be increased not only by decreasing the temperature during irradiation, but also by decreasing the temperature *after* the irradiation treatment. In fact, the one experiment which gave the greatest difference between the sets at high and low temperatures was one of this sort.

On the basis of this information, certain tentative conclusions can be drawn: (1) that class of induced changes in Drosophila known as induced lethal mutations, known to consist of a variety of different types of gene and chromosomal alterations, does not fit into the thermodynamic concept of the process of gene mutation. It may be questioned whether any of the so-called "gene mutations" in Drosophila are evoked by a chemical reaction that takes place at or near the gene immediately after the absorption of the individual quantum. This question is fortunately open to a direct attack. (2) Since the reactive agents produced by the absorption of an X-ray quantum are generally considered to be available for chemical transformations only a fraction of a minute after the impact of the quantum (although recent data from the study of radio-chemical reactions suggest that this may not be completely true) the effect of the temperature shock after irradiation in increasing the mutation rate may perhaps be referred back to the mutation process itself. Thus the lower temperatures might be inhibiting some sort of "back-reaction," which at higher temperatures returns a certain proportion of potential mutations to their original state or to one not detectable as a mutation. This phenomenon has been observed for other types of radiation changes, as chromosomal breaks.

DISCUSSION

MR. GEORGE E. MACGINITIE: (California Institute of Technology): Were these temperatures lowered and raised at the same rates in all experiments?

DR. NOVITSKI: Yes. There are so many variables in the problem that that is one thing we tried to keep constant. The rate of lowering was brought about by exposure to a cold air blast and the temperature rise by ordinary exposure to room temperature so that the lowering was much faster than the rise.

DR. SPIKES: If I might be permitted to shift the emphasis from the genetic viewpoint for a moment, can you tell anything about the relative fertilizing power of the sperm after these three treatments? That is, where they received the same quantity of radiation but are treated differently. Is there any way of telling if you have a difference in fertilizing power of the sperm?

DR. NOVITSKI: Ordinarily the supply of sperm is far in excess of the demand, and if any sperm are knocked out because of the lost fertilizing capacity, they are undoubtedly replaced by those which can fertilize the egg. There is an effect on the viability of the individuals which result from fertilization with sperm which have been treated in this way, but here the effect is a chromosomal one and not physiological. There are some rather interesting results which bear on the more general problem of fertility. When we first started, it was decided that a temperature as low as possible should be used provided it did not interfere with fertility. A fairly large series was made up with flies, both female and male, subjected to low temperatures for quite a long period of time. It was discovered as long as a male or female survived it could then be mated and would show no infertility. We then took fertilized females and subjected them to low temperature during the irradiation. Oddly enough the animals proved to be sterile, and a little work on the point showed that what was happening was that the sperm which were stored inside the females were very susceptible to the cold treatment, whereas the same treatment had no effect on the sperm in the males or on the ova of the females.

DR. NORMAN H. HOROWITZ (California Institute of Technology): Do you know anything about the cytological effects of these treatments?

DR. NOVITSKI: That involves the analysis of large numbers of different lethals to determine whether or not in the cold treatment some one type was favored over another. I think I was deliberately vague on the definition of a lethal mutation; probably a large number of them are associated with chromosomal aberrations. It is known that a low temperature treatment increases the frequency of X-ray induced chromosomal abnormalities, but the correlation with lethals remains to be done.

DR. RAY D. OWEN (California Institute of Technology): Do you have any X-ray dosage data connected with this temperature treatment? If the situation were different from that normally studied without temperature treatment, might one detect it in terms of dosage curves?

DR. NOVITSKI: The dosage data for the two doses used, 1800 and 3600 r-units, show that the increment of effect after treatment at low temperatures is approximately proportional to the effect without such treatment. This relation does not appear to give any specific information about the nature of the differences in the two cases.

CHAIRMAN ENGLE: I take it nobody has studied cytologically the effects of a given standard dosage plus low temperatures on these flies to see if, at a certain temperature, you get differential destruction or disturbance of stages of spermatogenesis.

DR. NOVITSKI: No, as far as I know that has not been done. Drosophila is probably not the best animal to do it on.

DR. PAULING: Perhaps I should draw something on the board. I have been trying to understand how a mutation occurs and trying to get evidence about it by the simple method of asking geneticists what was going on. The problem that bothers me in general about reproduction is that I would not expect the use of a template for the formation of a replica to be perfect, 100 per cent. perfect. In the formation of antibodies with an antigen as the template great fluctuations are observed in the nature of the product, so a gene too, I would think, would not produce an exact replica of itself but something in the neighborhood; then, in the course of a long period of time a gene would settle down to such a configuration as would be most effective in producing a replica of itself. I might represent that by a curve of energy, although the property is somewhat different from energy (Fig. 1). Nevertheless, energy or stability is related to this property of being able to produce a replica of itself. A gene in the wild type of an organism might be represented by a point, A, near the minimum of this curve. Although there are fluctuations when such a gene produces a replica, A′ or A″, in the next mitosis, there is a good chance that a better replica of the original gene will be produced, nearer the bottom of the curve. The gene would thus tend to stay in the valley. If we continue drawing the curve, there may be another valley over here somewhere at B, and on irradiation perhaps you get not by cell division but by damage to the gene a changed gene represented by a point such as C. When the next cell division occurs in the process of reproduction you have perhaps nearly equal chance of this point moving over toward B or of moving back toward A. There is some chance of getting over the top of the hill and starting down to B, which would represent the lethal mutation, hence there would be, after the damage due to irradiation, a probability factor as to which direction the gene would go. A certain number of the new genes would be mutants, and a certain number would be the normal ones as a result of cell division.

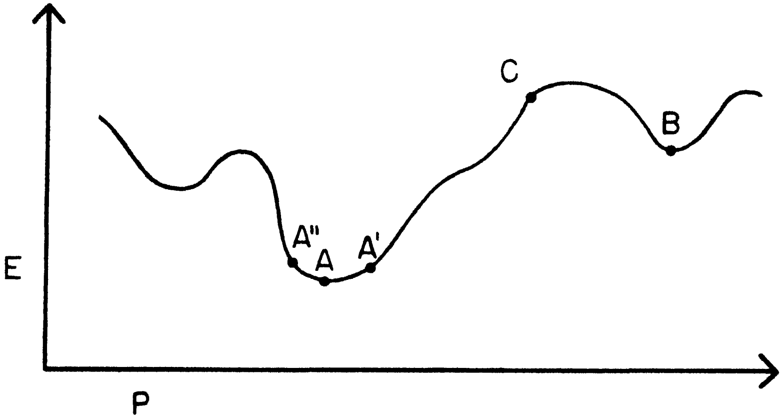

FIG. 1. Curve to illustrate manner of occurrence of a mutation. *E*, energy or other property related to self-duplicating power of a gene; *P*, parameter describing configuration of the gene. See discussion by Pauling in text.

Now what would be expected to happen without cell division? We have the damaged molecule, C, in a sort of unstable state where it has been left after the impact of the radiation. Thermal agitation would have the effect on it of tending to cause it to " mosey " down the hill toward the normal type A. Obviously, the resultant effect of change of temperature would be determined by the relative rates of the reactions of repair of the damaged gene by thermal agitation and of cell division. It seems to me that this idea offers the possibility of a rational explanation of these experiments and perhaps of other radiation experiments.

Dr. Novitski: When you say that it " moseys " down the hill, you imply that there is a fairly long time period involved?

Dr. Pauling: The time required to get down this hill would be of the order of magnitude of the time of molecular motion.

Dr. Novitski: I have experiments underway now which will give indications of the times involved at which this hypothetical " back reaction " can occur.

Dr. Erik Zeuthen: It is interesting that a sudden decrease in temperature just after the radiation is over may cause genetic damage, which might otherwise have been repaired, to show up. In the field of comparative physiology it has been shown that the immediate effect of a steep drop in body temperature is a " too low " energy metabolism. I say " too low " because during the following hours or days the energy metabolism again increases and becomes steady on a much higher level—at the same low temperature. Do you think this has any bearing on your problem? Metabolic energy might be involved in a repair process.

Dr. Novitski: I think that is a very good point, but our results indicate that if the period of time at the low temperature after the irradiation is of the order of half an hour, it produces much less effect than if the animals are kept down for fourteen hours.

Dr. Pease: I like your general notion, if I understand you correctly, that probably gross chromosome aberrations are most important in this effect. I wonder again if we can't interpret this in terms of gelation. I am thinking of a few data I never published which I collected years back on the effects of temperature coupled with high hydrostatic pressures. The data were not very significant until you got down to zero degrees or very close to it, but then, all of a sudden, the plasmagels became unduly sensitive to pressure. Apparently there was some sort of a complex and subtle effect on, shall we say, the steric relations of the protein molecules. Conceivably this would make them particularly sensitive to irradiation.

Dr. Novitski: That could be one of the finer aspects of the problem which I broadly termed a "physiological effect." I would like to point out that as far as chromosomal breakage itself is concerned, there has been a good deal of argument about the relation of this sort of mutation to gross chromosomal changes, and Fano has provided a very convincing argument to show that these lethal mutations should not be interpreted as the results of chromosomal breakage, but must have another origin, in part, at least.

Dr. Giese: I thought it might be interesting to point out a contrast here between the effect of irradiation and temperature on mutation and on activation. If one keeps irradiated unfertilized eggs at room temperatures most of them die; only at low temperatures does one obtain activation. Destructive processes are accelerated at high temperatures, death being the result. With mutation, on the other hand, the high temperature results in repair. However, in sea-urchin eggs repair is a result of active metabolism. Thus irradiated sea-urchin eggs can be stored for long periods of time after radiation. If they are inseminated at different times, the retardation per given dose of radiation is not decreased and apparently the repair process, whatever it is, is extremely slow, not showing up even eight hours after irradiation. In other words, until fertilization is accomplished and the various reactions which occur in the developing egg are stated, there doesn't seem to be much repair. The same thing can be seen in experiments with protozoa. Recovery from the effects of radiation is gradual and occurs only if the animals are fed and carrying on active metabolism.

Dr. Spikes: In confirmation of what Dr. Giese said, we found that the effect of ultraviolet light in suppressing the fertilization membrane persists for a long time after ultraviolet radiation. We kept the unfertilized eggs sixty-five or seventy hours and apparently there was no recovery at all from ultraviolet light.

Dr. Schechtman: We have quite a number of people interested in immunology here. I would like to ask whether, in the case of the rabbit or another animal if anybody present has heard of studies indicating that changes in the temperature of an animal result in qualitative differences in the antibodies, or are there only quantitative differences? I wonder if anyone knows of any studies which have a bearing on this idea.

Dr. Emerson: There was some work done by a student in this laboratory using fish which could be kept at low temperatures. More recent work has been done on fish and frogs. That has all been done on the quantities of antibodies and not characteristics. The most recent work showed if fish are kept at a low enough temperature they produce no antibodies. If they are kept a number of weeks and then brought back to higher temperatures, they produce antibodies as they would have to start with.

PROPERTIES OF FERTILIZIN AND RELATED SUBSTANCES OF EGGS AND SPERM OF MARINE ANIMALS[1]

DR. ALBERT TYLER

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CALIFORNIA INSTITUTE OF TECHNOLOGY

INTRODUCTION

SINCE I have recently published a rather detailed review of the work on fertilizin and related substances (Tyler, 1948) I shall discuss here only a few points on which we have some additional information. Some of the present audience may not be acquainted with this work and others may not recall very well the general nature of the reactions with which we are concerned. So I shall first review briefly, with illustrations, the action of these substances and indicate what role they are considered to play in the process of fertilization. Following this, I shall discuss their relation to the tissue- and species-specificity of fertilization, their location in the gametes and finally I shall present some information concerning their chemical properties.

F. R. Lillie (1913) demonstrated that the egg-waters of the sea urchin Arbacia and of the polychaet annelid Nereis caused an agglutination of the homologous spermatozoa and used the term fertilizin to designate the active agent in the egg-water. Fertilizins have since been obtained from eggs of various species of invertebrates and have recently been reported to occur in eggs of vertebrates too (see review by Tyler, 1948). We have demonstrated some time ago that fertilizin is derived from the gelatinous coat of the egg of the sea urchin and of other animals. The gelatinous coat slowly goes into solution as the eggs stand in water and yields a solution of fertilizin. Various extraction procedures will dissolve the gelatinous coat rapidly and give concentrated solutions of fertilizin. Our tests have shown no other substance of large molecular size, apart from fertilizin, to be present in the gelatinous coat.

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[1] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.

The substance on the sperm with which fertilizin reacts is termed antifertilizin. Some years ago Frank (1939) and I (1939) were able to extract this from sperm of the sea urchin and of other animals. There is also, within the egg, a substance that has properties similar to the antifertilizin which is obtained from sperm. The finding (Tyler, 1940) of such a substance within the egg that can react with a substance on the surface of the egg, and similar findings with other types of cells, have given rise to a so-called auto-antibody theory of cell structure, growth, and differentiation. This concept has been previously presented in some detail (Tyler, 1947) and will not be reviewed here. Another substance which is obtained from the sperm is a lytic agent. We have extracted this from sperm of various mollusks. Similar lytic agents have been obtained from sperm of fish, frogs, and mammals. The substance called hyaluronidase, which is derived from sperm of mammals, belongs to this general group.

ACTION OF FERTILIZIN, ANTIFERTILIZIN, AND LYSIN

Fig. 1 illustrates the agglutination reaction with sperm of the keyhole limpet. Fig. 1*a* shows a Syracuse dish with a suspension of untreated sperm. Fig. 1*b*, 1*c* and 1*d* show the reaction at 15 seconds, 30 seconds and ten minutes after addition of a solution of the fertilizin obtained from the eggs of this species. The sperm agglutinate in quite compact masses.

Fig. 2 shows the microscopic appearance of some of the agglutinates. In dense suspensions spherical masses of sperm are usually formed. Very often each sphere is surrounded by a complete or incomplete shell of sperm heads attached to the main mass by their tails.

Fig. 3 shows higher power photographs of small agglutinates. I am always intrigued by Fig. 3*b* which shows a clump of heads lined up like chromosomes on an equatorial plate and the tails united at their ends like spindle fibers. It certainly looks like a mitotic figure, but no matter how many times I show the figure, it never divides. I have discussed the question of head and tail agglutination in an

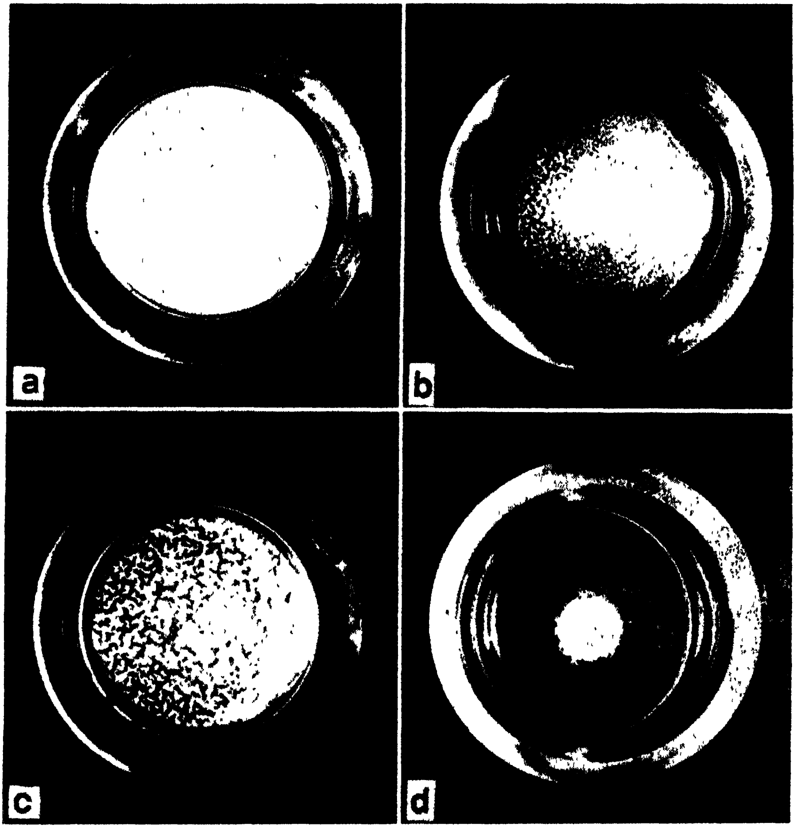

FIG. 1. Macroscopic appearance of agglutination reaction in the keyhole limpet, *Megathura crenulata*. Photographed in Syracuse dishes, × ⅘. *a*, Untreated sperm suspension (ca. 2 per cent.); *b*, *c*, and *d*, 15 seconds, 30 seconds and 10 minutes, respectively, after addition of solution of fertilizin.

early publication and shall not take the time to go over the subject again here.

When a solution of antifertilizin, prepared from sperm or from the interior of the eggs, is added to a suspension of intact eggs, then the eggs are agglutinated. Fig. 4 shows the microscopic appearance of the reaction when antifertilizin is added to a suspension of eggs of the sea urchin.

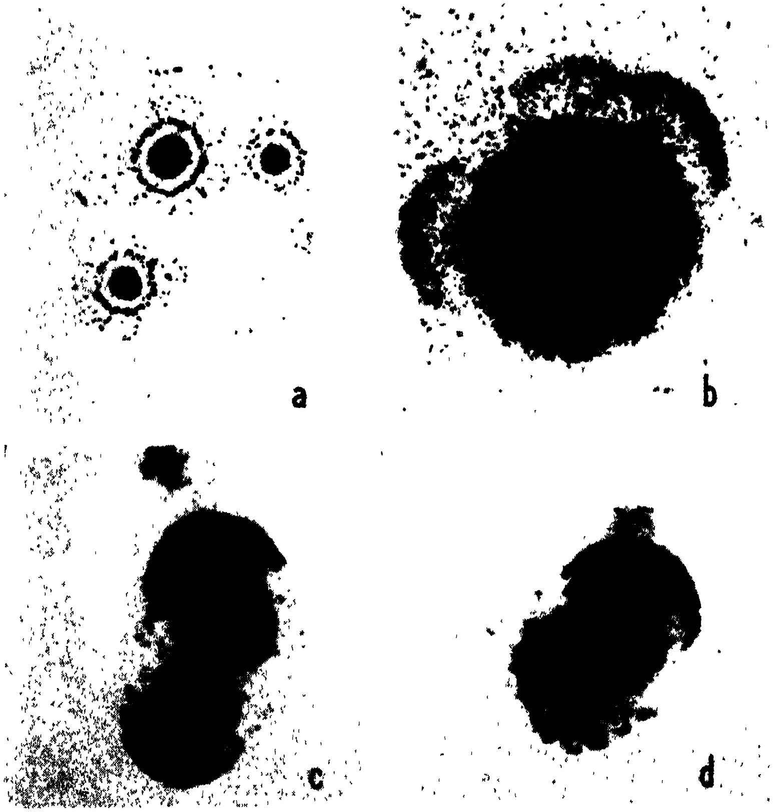

FIG. 2. Photomicrographs of agglutinated sperm of the keyhole limpet, *Megathura crenulata*. *a,* Three agglutinates formed in a moderately strong solution of fertilizin, showing spherical shell of sperm heads surrounding central mass of sperm; × 60. *b,* An agglutinate formed in a strong solution of fertilizin, showing incomplete shells of sperm heads attached to main mass by the ends of the tails; × 200 *c* and *d,* Fusion of two agglutinates; *d* was photographed 15 seconds after *c;* × 100

Fig. 5 shows some microscopic details of the action of antifertilizin on eggs of the sea urchin. Very soon after the addition of a strong solution of antifertilizin a precipitation membrane is formed on the surface of the gelatinous coat

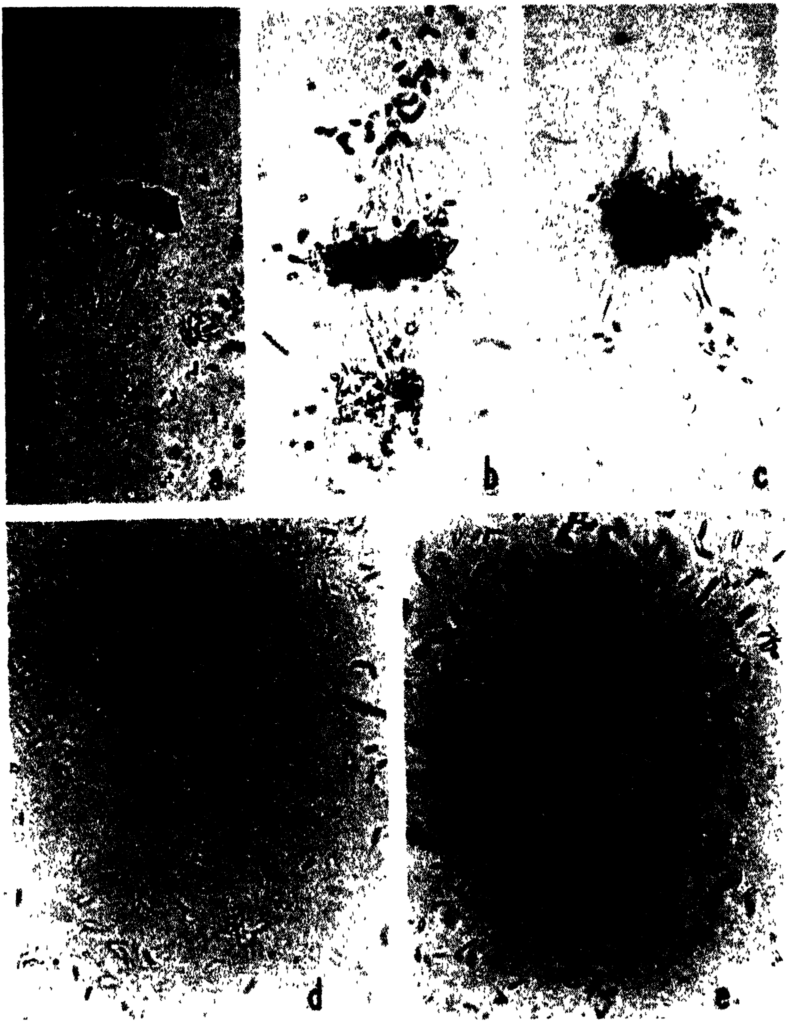

FIG. 3. Photomicrographs of sperm of *Megathura crenulata*, × 300. *a*, *b*, and *c*, Show union by heads and by end pieces of tails in strong, moderate and weak fertilizin solutions, respectively. *d* and *e*, Show head aggregates originally present in sperm suspension.

of the egg. The membrane thickens and contracts. In a strong antifertilizin solution it finally contracts to the surface of the egg and is then practically indistinguishable from the egg surface (Fig. 5*d*). This will take place in as short time as a minute in highly concentrated solutions. Some workers abroad interpreted the disappearance of the gelatinous coat as due to the action of a dissolving agent in the sperm extract. However, it is easy to demonstrate, by shaking off the gelatinous coat and adding the sperm extract to the isolated coat, that it doesn't disappear. It persists in the form of the precipitation membrane. Figs. 5*e* and *f* are two photographs, in the time interval of 19 minutes showing the precipitation membrane shrinking to the surface of an egg and, in an adjacent isolated jelly hull, the persistence of the precipitation membrane. Figs. 5*g* to *j* illustrate the effect of antifertilizin on fertilized eggs. Here, too, a precipitation membrane forms, and it is interesting to note that it resembles the fertilization membrane that is present below it.

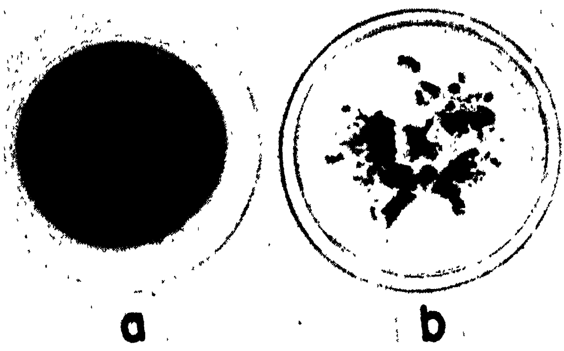

FIG. 4. Eggs of the sea urchin, *Strongylocentrotus purpuratus*; photographed in Syracuse dishes, × 7/10. *a*, Untreated egg-suspension. *b*, 15 minutes after addition of a solution of antifertilizin.

Fig. 6 illustrates the action of the lytic agent obtained from the sperm of mollusks; in this case from the keyhole

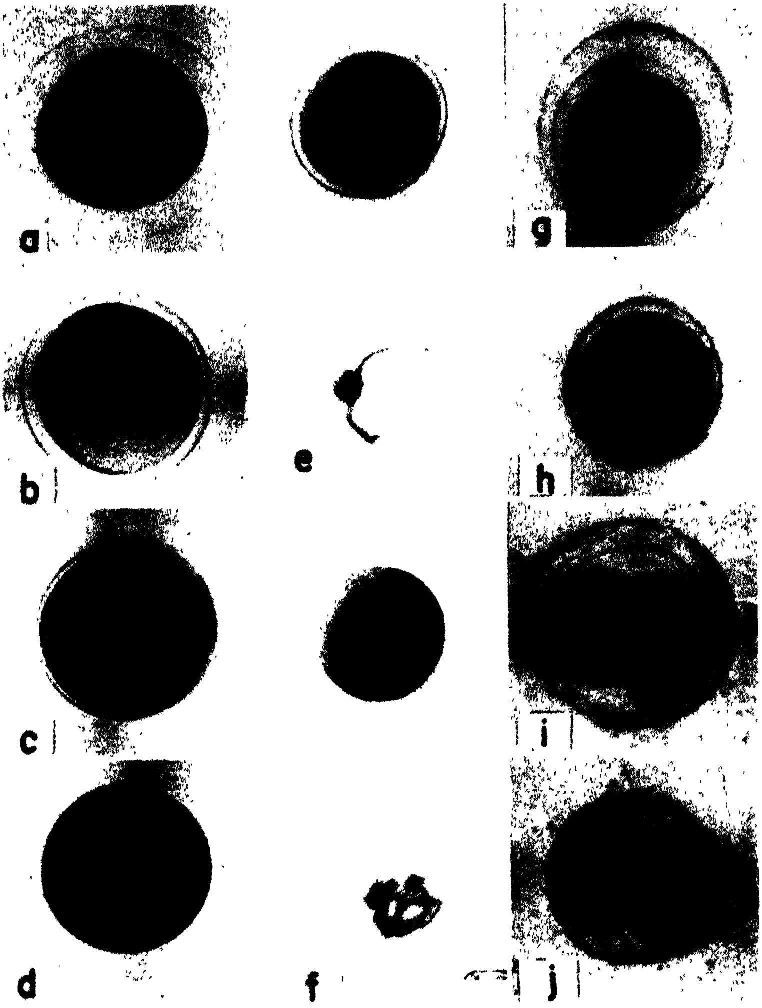

Fig. 5. Eggs of the sea urchin, *Lytechinus pictus*. *a, b, c,* and *d,* Successive pictures of the same egg at about ½ minute intervals after addition of a solution of antifertilizin, showing formation of the precipitation membrane and its contraction to the surface of the egg; × 400. *e* and *f,* Successive pictures of the same egg and an adjacent isolated jelly-hull at 1 minute and 20 minutes respectively after addition of antifertilizin, showing the persistence of the material of the isolated jelly-hull at the time when the precipitation membrane has contracted to the egg's surface and has become indistinguishable from it; × 350 *g* and *h,* Successive pictures of the same fertilized egg at 1 minute and 3 minutes after addition of antifertilizin. *i* and *j,* Successive pictures of a fertilized egg in the two-cell stage at 1 minute and 4 minutes after addition of antifertilizin; × 350.

limpet. The unfertilized egg of the keyhole limpet normally has a tough membrane on the surface. When the eggs are shed into the sea water the membrane separates somewhat from the surface of the egg proper. Upon the addition of the lysin-containing solution of sperm extract of the keyhole limpet this membrane swells and thins out and finally disappears. This can take place in as short a time as forty-five seconds in a strong solution of lytic agent. Incidentally, the membrane is a rather tough affair and rather resistant to ordinary chemical treatment. Mr.

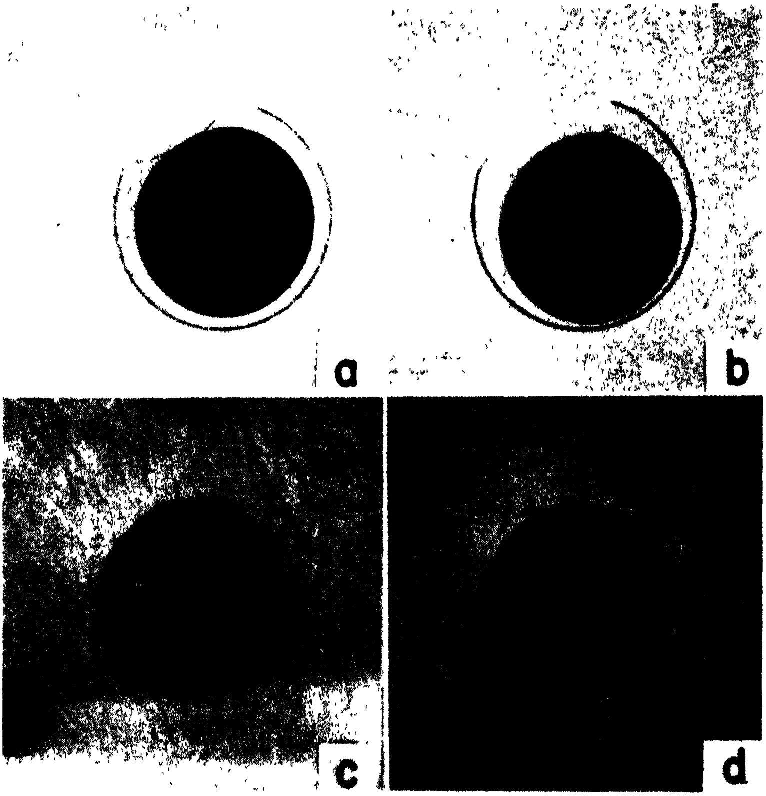

FIG. 6. Photomicrograph of an egg of *Megathura crenulata* at: *a,* 1 minute; *b,* 1¾ minutes; *c,* 2½ minutes; *d,* 3¼ minutes after addition of a sperm extract containing the egg-membrane lysin. × 200.

Krauss, who has been working with it recently in our laboratory, informs me that the eggs can be left in eight normal hydrochloric acid overnight with no noticeable effect on the membrane.

SPECIFICITY

One of the problems of fertilization in which we are interested is the species- and tissue-specificity of the process. In this connection we have made extensive tests of the cross-reactivity of fertilizin and antifertilizin of various species of sea urchins and starfish and have compared the degree of cross-reactivity with the degree of cross-fertilizability of the same species of animals.

First I should remark that we have some earlier evidence concerning the role of these substances in fertilization. For example, there are experiments in which the gelatinous coat of the egg is removed and experiments in which we deprive the sperm partly of the antifertilizin or treat them with antibodies against antifertilizin. These experiments show that fertilizin serves as an aid to fertilization, but only when it is present on the surface of the egg, as the gelatinous coat. If a concentrated solution of fertilizin is added to a suspension of eggs and fertilization is attempted, or if sperm are treated with fertilizin and used for insemination, fertilization is inhibited and may even be completely blocked. Evidently when the interaction of the sperm and fertilizin takes place away from the egg and is completed before the sperm reaches the surface of the egg, the sperm are then unable to combine with the egg. This is not due to the sperm being tied up in the agglutinates, because agglutination is spontaneously reversible in the sea urchin. After a period of agglutination, the length of which is dependent upon the concentration of fertilizin, the sperm separate, appear quite normal and swim actively about. Nevertheless, they are incapable of fertilization.

While eggs that have been deprived of their gelatinous coat show, in general, a reduced fertilizability, they are still fertilizable. This does not, however, mean that fertilizin, as the gelatinous coat, serves only as an aid to fertilization and is not an essential link in the process. To decide whether or not it is essential for the process it would be important to remove the fertilizin completely. However, this does not appear to be readily feasible since, at the surface of the egg, where the gelatinous coat joins the egg, there is evidently a layer of fertilizin that is not removed by the treatments used to remove the gelatinous coat and which apparently can not be removed without disruption of the egg.

Similarly, the experiments on partially depriving sperm of antifertilizin show that their fertilizing capacity is impaired without corresponding impairment of their activity or their respiratory rate. Specific antibodies against antifertilizin can also impair the fertilizing power of the sperm without visible effect on the sperm.

In comparing the degree of cross-reactivity of these substances with the degree of cross-fertilization, there are a great many ways one may proceed, and I can, in a limited time, simply indicate one type of comparison and a sample of the data we have obtained. In the first place all the species of echinoids that we have tested here cross-fertilize. One can specify a degree of cross-fertilizability since the percentage of cross-fertilization that is obtained depends upon the amount of sperm employed for insemination. Of course, the percentage of fertilization obtained within the species, which we may call iso-fertilization, also depends upon the amount of sperm employed. This is apart from the fact that only one sperm enters the egg. One sperm is, of course, sufficient to fertilize an egg but because of several known, as well as unknown, factors, many more than one spermatozoon per egg must be present in the inseminates. As the amount of sperm employed in iso-insemination is increased the percentage of fertilization increases from zero to 100. In cross-fertilization the same thing occurs, except that it will require much higher concentrations of sperm and, in some cases, we may not be able to get the sperm concentrated enough to fertilize all the eggs. The degree of cross-fertilizability of a particular pair of species can be expressed in terms of the amount of sperm required to give a certain percentage of fertilization below 100 per cent., say, five per cent., under standard conditions. Due to variations in different lots of animals, changes in activity of the sperm with time and similar factors it is advisable that as many different tests as possible be performed at the same time, or, at least, that there be a standard of comparison, for example, the iso-inseminations, in each set of tests. The degree of fertilizability of a particular combination can, then, be expressed by the figure for the number of times that a unit volume of standard sperm suspension is diluted in order to give the end point percentage of fertilization.

In Table 1 the average values for some of the data of this sort, accumulated for four species of echinoids, is presented as the upper figures in each pair of rows. The lower figures in each pair of rows give averages of the titers for the agglutination of sperm by fertilizin among the same species. No matter what basis of comparison we have used for these figures, or other data of this sort, we can find no very close correspondence between the degree of cross-fertilizability and the degree of cross-agglutination. We can interpret this to mean that the specificity of the fertilizin-antifertilizin reaction does not, in itself, account for the specificity of fertilization. This is not surprising, since there are un-

TABLE 1

(COMPARISON OF CROSS-FERTILIZATION WITH CROSS-AGGLUTINATION AMONG ECHINOIDS)
(THE UPPER FIGURES OF EACH PAIR OF ROWS REPRESENT THE NUMBER OF TIMES THE SPERM SUSPENSION IS DILUTED IN GIVING THE END POINT VALUE (2 PER CENT.) OF FERTILIZATION UNDER CERTAIN STANDARD CONDITIONS. THE LOWER FIGURES ARE THE AGGLUTINATION TITERS IN TERMS OF THE HIGHEST DILUTION OF FERTILIZIN SOLUTION THAT GIVES VISIBLE AGGLUTINATION)

Eggs or Fertilizin of	Spermatozoa of			
	S. purp.	S. fran.	L. pictus	D. excent.
Strongylocentrotus	8000	8	1	5
purpuratus	512	8	64	4
Strongylocentrotus	1	600	1	2½
franciscanus	0	512	4	0
Lytechinus	2	4	850	2½
pictus	64	82	64	8
Dendraster	1½	40	2	4400
excentricus	4	2	1	128

doubtedly many other reactions involved in the process of fertilization. Other, less extensive, tests with the lytic agent obtained from sperm of various mollusks show a broad cross-reactivity for this material which, likewise, can not by itself account for the species-specificity of fertilization.

On the other hand, emphasis should be placed upon the fact that where cross-fertilization occurs there is generally cross-agglutination. In Table 1 the only exception to this are the cases of *S. franciscanus* eggs or fertilizin with *S. purpuratus* and *D. excentricus* sperm. But these are only apparent exceptions, because we have found, by absorption experiments, that *S. purpuratus* sperm and *D. excentricus* sperm combine with *S. franciscanus* fertilizin. On the other hand, tests with various species of asteroids and other more distantly related animals, not listed in the table, have given neither cross-fertilization, cross-agglutination or absorption of the fertilizin by the foreign sperm.

Concerning tissue-specificity it has been shown by Lillie, and confirmed by various workers including ourselves, that no tissues other than the eggs and sperm yield fertilizin and antifertilizin.

We may, then, conclude that both the tissue and species specificity of fertilization is based, at least partly, on the specificity of the fertilizin-antifertilizin reaction.

We have also prepared antisera in rabbits against the various substances, including fertilizin and antifertilizin, obtained from the eggs and sperm of several species of echinoderms, annelids and mollusks. While a considerable amount of data has been accumulated, much more work will be required before a proper analysis can be made. I shall indicate briefly some of the results that have been obtained. In the first place the results show that the rabbit does not distinguish very closely the substances of one species of echinoid from those of another species. For example, as shown in Table 2, there is considerable cross-reactivity between an antiserum against the sperm extract of one species and the sperm of other species. Upon absorption one can demonstrate the presence of specific antibodies for different

TABLE 2

AGGLUTINATION TITERS OF RABBIT ANTISERA *vs.* THE ANTIFERTILIZIN OF THE SEA URCHINS *Strongylocentrotus purpuratus* AND *Lytechinus pictus*, TESTED ON SPERM OF VARIOUS SPECIES OF ECHINODERMS

Sperm of:	Antiserum vs. antifertilizin of	
	S. purp.	L. pictus
Echinoids		
Strongylocentrotus purpuratus	512	256
Strongylocentrotus franciscanus	512	128
Lytechinus pictus	128	128
Lytechinus anamesus	128	128
Lovenia cordiformis	8	16
Dendraster excentricus	16	16
Asteroids		
Patiria miniata	0	0
Pisaster ochraceous	0	0
Astropecten armatus	0	0

species, and the absorption tests indicate that there are at least two kinds of antibodies produced in response to the injection of what may represent a single molecular species. In the experiments listed in Table 3, for example, we have injected the rabbits with an electrophoretically homogeneous solution of antifertilizin of *S. purpuratus* and then absorbed the antibodies with the foreign sperm. That still leaves antibodies in the serum that react with the original sperm. If the antiserum is absorbed with homologous eggs it still agglutinates homologous sperm, but the titer is reduced. If absorbed with blood, it is reduced likewise, but in both of the latter cases the foreign sperm is agglutinated with unchanged titer. If it is absorbed with a mixture of the homologous blood and foreign sperm, then the antibodies against the homologous sperm are completely removed. In other words, after injection of antifertilizin of

TABLE 3

AGGLUTINATION TITERS OF AN ANTISERUM *vs.* ANTIFERTILIZIN OF *Strongylocentrotus purpuratus* AFTER ABSORPTION WITH VARIOUS KINDS OF CELLS

Absorbed with:	Tested on sperm of:	
	S. purp.	L. pictus
Unabsorbed	512	128
L. pictus sperm	128	0
L. pictus eggs	512	128
L. pictus blood	512	128
S. purp. eggs	256	128
S. purp. blood	256	128
S. purp. blood + L. pictus sperm	0	0

one species, there are antibodies formed that react with sperm of various species and also antibodies formed that react with other tissues (eggs and blood cells) of the same species but not with the corresponding tissues, the eggs and blood, of the foreign species. It might appear, then, that the injection of a single kind of protein molecule can give rise to antibodies that react with the same kind of protein in other species and also to antibodies that react with different proteins of the homologous species. Results of this sort that have been reported in the immunological literature have, in general, been interpreted as due to the presence of other protein of the homologous species as contaminants in the preparation used as the immunizing antigen. Further work will be required to determine whether or not this is true in our experiments as well as in some of the recent experiments with "purified" serum proteins that have given somewhat similar results.

LOCATION OF ANTIFERTILIZIN AND OF THE LYSIN IN THE SPERMATOZOON

Mention was made earlier of the fact that fertilizin is derived from the gelatinous coat of the egg. We have also attempted to determine from what region of the sperm the antifertilizin is derived. To investigate this we have made use of the high magnification that is provided by the electron microscope. If sea urchin sperm is treated in such a way as to extract some antifertilizin, for example at pH 3.5, the head of the sperm between the acrosome and midpiece becomes spherical and slightly swollen, as shown in Fig. 7*b*. More drastic extraction results in further swelling and diffuseness, as in Fig. 7*c*. The acrosome is still present but covered by the swollen part of the head in this picture. Upon still further extraction the acrosome, mid-piece and tail remain intact, but the intervening region of the head becomes quite swollen and diffuse, as shown in Fig. 7*d*. We conclude that the antifertilizin is located laterally around the head, in the region between the acrosome and mid-piece.

We have also attempted to determine what part of the

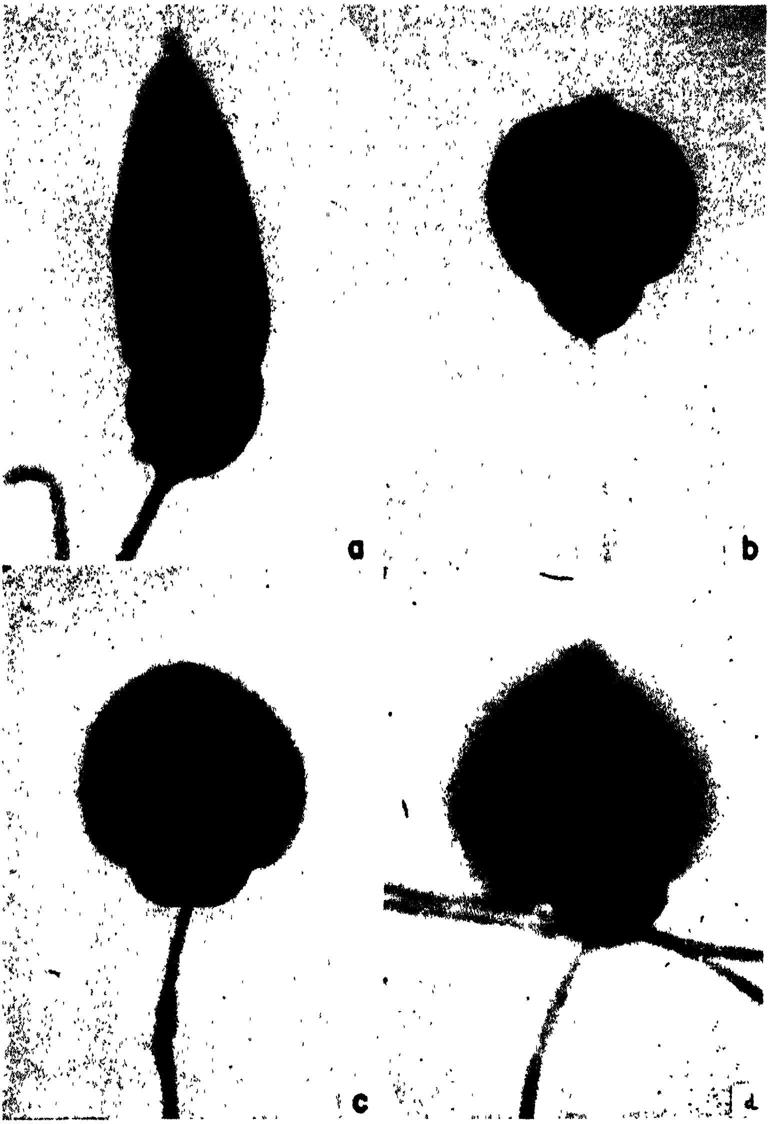

FIG. 7. Electron micrographs of sperm of the sea urchin, *Lytechinus pictus*, showing changes upon extraction of antifertilizin; × 30,000. *a*, Control, unextracted sperm. *b*, Extracted at pH 3.5. *c*, Extracted at pH 3.0. *d*, Extracted at pH 2.8.

sperm of the keyhole limpet contains the lytic agent. About this we are not quite as certain as the pictures of Figure 8 may lead you to believe. When we extract keyhole limpet sperm in such a way as to obtain the lytic agent, we find the acrosome to be affected. Thus Figs. 8*b* and *c* show striking effects on the acrosome which may be broken down partially

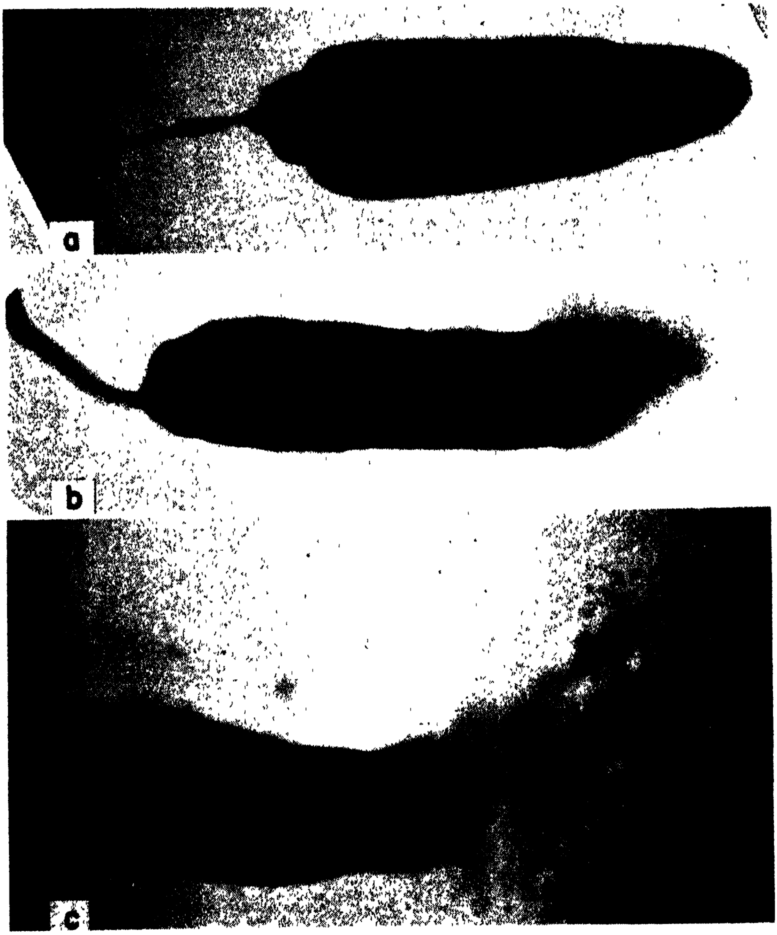

FIG. 8. Electron micrographs of sperm of the keyhole limpet, *Megathura crenulata*, showing breakdown of acrosome possibly correlated with liberation of egg-membrane lysin *a*, Control sperm from a suspension in ordinary sea water. *b* and *c*, Sperm from a suspension in sea water at pH 9. × 30,000.

or quite completely after an extraction with weak alkaline sea water that yields a solution of the lysin. The reason that I say we are not quite certain about this is that we find this sort of broken-down acrosome in untreated sperm too. While untreated sperm also yields some lysin in the supernatant, it is very difficult to make counts of the various types of sperm under the electron microscope in order to determine whether or not the number of disintegrated acrosomes might account for the amount of lysin obtained from untreated suspensions.

Chemical Properties

A fair amount of information has now been accumulated concerning the chemical nature of these various substances; especially the fertilizin and antifertilizin of sea urchins. Both of these can be prepared in electrophoretically homogeneous form. The antifertilizin is an acidic protein, isoelectric at about pH 3 and having a rather typical nitrogen content of about 16 per cent. Table 4 lists some electrophoretic mobility data obtained with the antifertilizins of Echinocardium by Runnström in Sweden and of Lytechinus

TABLE 4

Electrophoresis of Purified Solutions of Antifertilizin of *Echinocardium cordatum* (Runnström et al., 1942) and of *Lytechinus pictus* (Tyler, Unpub.)

pH	Buffer	Mobility in cm.^{2}/sec./volt × 10^5	
		Echinocardium	Lytechinus
4.0	acetate	3.0	
4.5	acetate		4.2
4.6	acetate	3.7	
4.9	acetate		4.8
5.0	acetate	4.1	
6.0	phosphate		6.1
6.9	phosphate	6.4	
7.3	barbital		9.3

TABLE 5

Method of Preparation and Initial Purification of Fertilizin of the Sea Urchin *Strongylocentrotus purpuratus*

Extract 1 vol. of washed eggs with 40 vols. of pH 3.5 sea-water; agglutination titer = ca. 1000.
Absorb on sea-water salts precipitated by 4 ml N/1 NaOH per liter.
Dissolve in 3.3% NaCl and dialyze.
Precipitate with 1¼ vols. 95% Alc.
Dissolve in saline; reprecipitate with alcohol or ammonium sulphate.
Yield = ca. 250 mg./liter.

by ourselves. The two sets of values show reasonably good agreement.

Fertilizin is, in some respests, a more interesting substance, chemically. Table 5 gives a simplified method for the preparation of electrophoretically homogeneous fertilizin and Table 6 lists data for the electrophoretic mobility at various pH's. As may be seen from the latter values the mobility changes very little as the pH is lowered from 8.6 down to 2.0. This means that fertilizin is a highly acidic substance. We now understand the basis of the highly acidic properties. Vasseur (1947), in Runnström's laboratory, has found about 25 per cent. of sulphate to be present, and we have confirmed this. Evidently we are dealing with a sulphuric ester, such as is found in chondroitin sulphate or mucoitin sulphate. We have also determined sedimentation constants of fertilizin preparations with a Pickel's analytical ultracentrifuge. It is relatively easy to obtain preparations, by a single careful extraction of the eggs, that are homogeneous in the ultracentrifuge. Fig. 9 shows the sedimentation boundary of one such preparation. This gives a sedimentation constant (S_{20}) of 6.3×10^{-13}, which, if the molecule were spherical, would correspond to a molecular weight of 82,000. After various precipitation procedures a large fraction of the material may become aggregated into larger particles which are poly-disperse in the ultracentrifuge.

TABLE 6

ELECTROPHORESIS OF A PURIFIED SOLUTION OF FERTILIZIN OF THE SEA URCHIN *Strongylocentrotus purpuratus*

pH	Buffer	Mobility in cm.^{2}/sec./volt $\times 10^5$	
		Descending arm	Ascending arm
8.58	barbital	16.7	16.6
8.58	barbital	16.7	16.2
6.75	phosphate		18.8
6.60	phosphate	19.5	19.7
5.65	acetate	17.4	17.8
3.76	acetate	17.8	18.5
2.00	phosphate		18.8

Table 7 presents some of the analytical data obtained with purified fertilizin of *S. purpuratus*. In the first place it should be noted that fertilizin gives both protein and polysaccharide reactions; so it belongs to the group of sub-

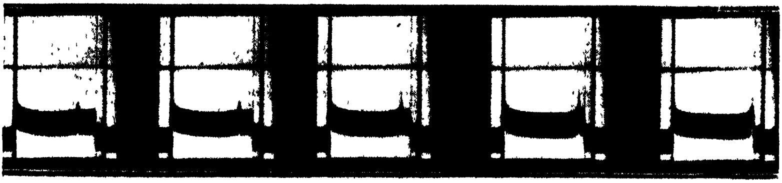

FIG. 9. Sedimentation diagram of a fertilizin preparation (pH 3.5 extraction and dialysis) of the sea urchin, *Strongylocentrotus purpuratus*, at 40,800 r.p.m and 6 cm. radius. The five photographs are taken at (reading left to right) 5, 21, 37, 53 and 69 minutes of centrifugation. The preparation shows a single boundary (peak) which sediments with an average s_{20} of 6.3×10^{-13} cm./sec./dyne/gm.

stances that are termed glycoproteins or mucopolysaccharides depending upon whether the protein or polysaccharide properties are considered to be strongest. It should, however, be emphasized that it does not appear to be possible to dissociate fertilizin into protein and polysaccharide fractions. We have made extensive attempts to do this and interpret the results to mean that protein and polysaccharide constituents do not exist as such in the molecule but rather that the various sugars and amino-acids are interlinked. As the figures of Table 7 show, values of about 25 and 20 per cent. have been obtained for reducing sugar and amino-acid respectively. These are minimum values, since a large amount of humin-residue is formed upon the acid hydrolysis of the fertilizin and an unknown amount of reducing sugar and amino-acid (tryptophane and perhaps tyrosine) is tied up in this residue. One of the sugars, that has been identified in the form of the osazone, is galactose.

TABLE 7

CHEMICAL ANALYSIS OF PURIFIED PREPARATIONS OF FERTILIZIN OF THE SEA URCHIN *Strongylocentrotus purpuratus*

	Per cent.
Nitrogen	5.6-5.8
Carbon	33.3
Hydrogen	5.5
Sulphate	28
Phosphate	0.06
Reducing sugar	> 25
Amino-acids	> 20
Glucosamine (?)	1.6
Galactose	pos.
Glucuronic acid	neg.
Pentoses	neg.
Mol. Wt. (as sphere)	82,000

Little, if any, real hexosamine is present, and tests for glucuronic acid and pentoses are negative. Upon paper chromatography of hydrolyzed fertilizin we find seven amino-acid spots. Table 8 lists the choices of particular amino-acids for each of these spots on the basis of their R_F values.

TABLE 8

CHROMATOGRAPHY OF A HYDROLYZED PREPARATION OF PURIFIED FERTILIZIN OF THE SEA URCHIN *Strongylocentrotus purpuratus* BY THE FILTER-PAPER METHOD OF CONSDEN, GORDON AND MARTIN (1944)

Spot No.	R_F values		Possible amino acid	R_F values	
	phenol	collidine		phenol	collidine
1	0.34	0	aspartic	0.30	0
2	0.43	0	glutamic	0.48	0
			serine	0.48	0
			a-amino-adipic	0.44	0
3	0.54	0	glycine	0.50	0
			threonine	0.57	0.08
			cystine	0.62	0
4	0.71	0	lysine	0.71	0
			alanine	0.70	0 01
5	0.82	0	histidine	0.77	0
			norleucine	0.84	0
			leucine	0.87	0
			arginine	0.87	0
6	0.85	0.09	tryptophane	0.80	0.12
			methionine	0.84	0.12
			phenylalanine	0.86	0.12
7	0.90	0.19	isoleucine	0.87	0.12

Sea urchin fertilizin belongs, then, to that group of sugar and amino-acid containing compounds that include such interesting substances as the human blood group substances. We have made some preliminary investigation of the composition of the fertilizins of different species of sea urchins. The present data show no qualitative difference between the two species *Strongylocentrotus purpuratus* and *Lytechinus anamesus*. It is known that antibodies with diverse reactivity may have the same chemical composition, the differences in specific reactivity being attributed to differences in shape of the molecule. The present data are too inadequate to indicate whether or not this may be true, too, of the fertilizins.

LITERATURE CITED

Consden, R., Gordon, A. H., and Martin, A. J. P.
1944. *Biochem. Jour.*, 38: 224–232.
Frank, J. A.
1939. *Biol. Bull.*, 76: 190–216.

Lillie, F. R.
1913. *Jour. Exp. Zool.*, 14: 515–574.
Runnström, J., Tiselius, A., and Vasseur, E.
1942. *Arkiv. Kemi, Mineral., Geol.*, 15A: No. 16, 1–8.
Tyler, A.
1939. *Proc. Nat. Acad. Sci.*, 25: 317–323.
1940. *Proc. Nat. Acad. Sci.*, 26: 249–256.
1947. *Growth*, 10 (Suppl): 7–19.
1948. *Physiol. Rev.*, 28: 180–219.
Vasseur, E.
1947. *Arkiv. Kemi, Mineral., Geol.*, 25B: No. 6, 1–2.

DISCUSSION

CHAIRMAN ENGLE: Thank you, Dr. Tyler. You have extraordinarily important work before you. We will give the chemists a chance to discuss this.

MR. MACGINITIE: Was there any indication of recovery of the sperm after they had been, not inactivated, but put out of commission by fertilizin?

DR. TYLER: No. In the sea urchin the agglutination reaction is a spontaneously reversible affair, such as it is, for example, with red blood cells agglutinated by influenza virus. When the agglutinates break up the sperm are perfectly normal in appearance and quite active. In fact, they are somewhat more active than previously, since there is an activating agent present in the egg water about which we don't know very much as yet. Nevertheless, they are incapable of fertilizing the eggs, and don't recover this ability on aging.

MR. MACGINITIE: What made me ask was because of the experiment done on mixing sperm with the eggs in the Urechis segmental organ or storage organ. There is no fertilization within the organ, but after removal of the mixture to sea water the sperm begin to recover and will fertilize eggs in time.

DR. TYLER: I think that is a very interesting and important finding in regard to fertilization. There has been a good deal of earlier work, as you know, on the so-called blood-inhibition of fertilization in sea urchins. Dr. Pequegnat recently went over that work again. Perhaps he would like to say a few words about it.

DR. PEQUEGNAT: At present it is possible to say a substance obtained from certain blood cells of the perivisceral fluid of several echinoderms (especially echinoids) is capable of blocking fertilization. As yet one can not say with certainty that the substance operates against the egg, but there is more evidence in favor of this opinion than for the opinion that it is the sperms which are inactivated. Assuming that the substance does act principally upon the egg, it is of considerable interest that the blocking effect is reversible. Thus, eggs previously exposed to the inhibitor and thereby rendered unfertilizable could be rendered fertilizable by simply washing them in several changes of sea water. At present I am attempting to determine the mechanism responsible for this blocking of the egg.

DR. TYLER: As I recall, you obtained it also from the tube feet of the animal. Is that correct?

DR. PEQUEGNAT: Yes. I found a very rich source of inhibitor to be certain yellowish bodies (presumably one type of amoebocyte) in the hypodermis of the oral tube feet. Although the generally held opinion regards these cells as being laden with waste products (at least one of which is very likely the inhibitor itself), one can not escape the conviction that such is not the case. For it seems very unlikely that a large concentration of waste products derived from another point in the body would be stored in the most active organs of the animal.

MR. MACGINITIE: The experiment I was referring to was the one in which the sperm was placed in the storage chamber and left there for a period of twenty-four hours. Then after they were taken out they appeared to be just as active as sperm could be but would slide all over the surface of an egg without attaching. After a period of an hour some would begin to attach, and from then on fertilization could take place.

DR. TYLER: The reason that I brought up blood inhibition in this connection is that it seems quite likely that the fluid within the storage organs is very much like the plasma, since these organs are in direct communication with the hemocoel. As Dr. Pequegnat has shown, the so-called blood inhibition is reversible upon washing the gametes in sea water. So it is conceivable that, in your Urechis experiments, the dilution occasioned by transfer of the mixture of eggs and sperm to sea water permits the inhibiting agent, which may be loosely absorbed on the surface of the gametes, to diffuse slowly away and thus become ineffective.

MR. MACGINITIE: you don't feel that it was the fertilizin or the contact with the egg, or that the activity of fertilizin had anything to do with it?

DR. TYLER: Of course I would like to interpret everything on the basis of fertilizin, but that temptation is just too great. I will have to resist it in cases like this until there is definite evidence that fertilizin is directly involved.

DR. HAAS: Hyaluronidase in snake venoms or in bacterial extracts is accompanied by other enzymes which we have designated as proinvasins. Are there any indications to suggest that the lytic substance in sperm might be associated with other proinvasin-like enzymes?

DR. TYLER: I can say it could be. The lytic agent has not, as yet, been really purified. So it is possible that two or more substances may be involved. One experiment which relates to the question consists in adding heat-inactivated sperm extract to fresh sperm extract. This does not result in increased activity. But this does not necessarily mean that only one substance is involved, since the other substances might also be heat-labile or the heat-labile component might be normally present in excess.

DR. HAAS: Would it be possible, for example, to add small amounts of snake venom to fertilizin and to obtain thereby an increased activity of the lytic substance?

DR. TYLER: I don't know whether we have any data that would properly answer your question. Mr. Krauss, do you know?

Dr. Max Krauss: In the work that I have been doing here with the egg membrane lysin in the keyhole limpet it is my impression, at this time at least, that there is no other substance which acts with it in the manner which you suggest.

Dr. Laties: How pertinent is the supposition that the areas found disrupted on the sperm as a result of extraction are necessarily the sources of the substance you are seeking?

Dr. Tyler: I presume that you are asking whether or not I have any rational basis for the conclusion other than my inference from the pictures. First, I think it is rational to assume that the antifertilizin is somewhere on the surface of the sperm by virtue of the fact you get agglutination by adding fertilizin. There have been claims of action at a distance in antigen-antibody reactions, such as in the work of Rothen, but these have not been substantiated and have been effectively contradicted by the recent experiments of Singer in the Chemistry Division here. Since we can safely assume that antifertilizin is on the surface we can interpret the figures as meaning that dissolving it off weakens the surface in such a way that that part of the head can swell. It is possible that other material might leak out of the inside of the head but, at least in our preparations, we don't find any indication of a second component.

Dr. Chambers: In regard to the role of fertilizin in fertilization, I am interested in your interpretation of this experiment. Arbacia eggs are placed in a non-electrolyte solution and then they are tested for nakedness of the protoplasmic surface by the oil-drop method. We found that the surface of the protoplasm met all the criteria of an entirely naked surface. When these eggs were placed in sea water containing sperm, they apparently went ahead and developed normally, having fertilized normally, of course with the absence of fertilization membrane. I am wondering what your interpretation would be.

Dr. Tyler: We, too, had found, as I indicated earlier, that sea urchin eggs can be fertilized after removal of the gelatinous coat. We have evidence to show that there is a layer of fertilizin left on the surface itself. I think Dr. Moore, this afternoon, will tell us some more about membrane inhibition.

Chairman Engle: If there are no more comments, we will continue. We shall proceed with further studies. Dr. Berg, of the University of California at Berkeley, will speak on "Some Effects of Sperm Extracts in the Mussel Mytilus."

(*Conference continued in next issue*)

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CONFERENCE ON PROBLEMS OF GENERAL AND CELLULAR PHYSIOLOGY RELATING TO FERTILIZATION. II.[1]

SOME EFFECTS OF SPERM EXTRACTS ON THE EGGS OF MYTILUS

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THIS is a short progress report on some of the work which I have carried out in the last few months. First, I would like to say a few words about Mytilus eggs as they have been used very little for experimental work and probably not many of you are familiar with them. *Mytilus edulis* is the common mussel that occurs abundantly in the San Francisco Bay. The animals are easily collected and if kept cold over night, they will usually spawn the next day.

Mytilus eggs look somewhat like sea urchin eggs. They are about 65 microns in diameter with closely adhering vitelline membranes, one or two microns in thickness. The membrane does not lift off after fertilization, as in the sea urchin, but remains closely adherent to the surface of the egg. The first cleavage is unequal with a large contact area between the two blastomeres, and consequently the blastomeres are not quite spherical due to this large flat contact area.

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[1] Sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949. Conference Chairman. Earl T. Engle, Columbia University, New York. Program Secretary, Albert Tyler, California Institute of Technology. Representative of the N.R.C., Deborah C. Leary, New Haven Hospital, New Haven.

My aim was to separate the blastomeres of early cleavage stages for physiological studies. The presence of the membrane makes it impossible to separate them by using a glass needle and although alkaline sea water will dissolve the membrane, it is injurious to the eggs. Recalling Dr. Tyler's discovery of an egg membrane lysin in Megathura sperm extracts, it occurred to me that such a lysin might be present in Mytilus sperm and this could then be used to remove the vitelline membrane. It was discovered that Mytilus sperm also contain a similar enzyme. If the sperm are concentrated, frozen, thawed and then centrifuged, the supernatant contains a substance capable of dissolving the membrane of Mytilus eggs. Ordinarily the membrane is so thin that it is difficult to see whether it has been dissolved; however, if the eggs are first plasmolyzed, the membrane stands out clearly and a sperm extract will cause its dissolution in a few minutes.

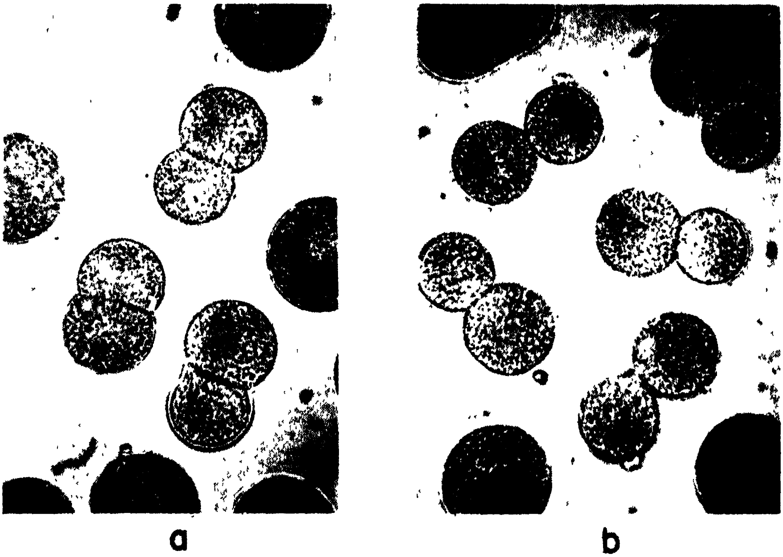

FIG. 1. (a) Normal appearance of blastomeres after first cleavage of Mytilus eggs. (b) Cleavage of Mytilus eggs in sperm extract showing the rounding up of blastomeres and reduction of contact area.

Eggs which have had the membranes removed by a short treatment with the sperm extract, however, still cleave in a normal fashion; the blastomeres are in very close contact and it is difficult to separate them. It was discovered that if the cleaving eggs were left in the sperm extract a striking thing happens. *The blastomeres become spherical and many of them become nearly or completely separated.* (See Fig. 1b). The blastomeres of any of the early cleavage stages can be separated by this method apparently without harmful effects. Also the polar lobe may be removed with ease.

In addition to perhaps being of future value as a method for separation of blastomeres, it is also of some interest that the sperm extracts contain a substance which has such an effect on the cleaving egg. The question is whether this "blastomere separating" substance is the same as the sperm lysin. As far as I could determine, the lytic effect of the sperm extract always parallels the blastomere separating effect. Whenever the lytic activity of a particular sperm extract is weak, the blastomere separating effect is also slight. Both effects of the sperm extract are destroyed after heating, although the Mytilus sperm lysin is much more resistant to heat than that of Megathura. There is still a slight activity after 10 minutes at 100° C. Again with heat treatment there seems to be a parallel inactivation of the sperm lysin and the disappearance of the blastomere separating effect. Perhaps the chemical methods used by Dr. Tyler would be helpful in deciding whether one or two substances are responsible for these effects.

It seems most likely that the sperm extract causes a separation of the blastomeres by dissolving the cement between them.

DISCUSSION

CHAIRMAN ENGLE: Thank you, Dr. Berg. Do you know anything about the nature of that membrane?

DR. BERG: You mean chemical properties? No, I don't, as I haven't carried out any chemical studies on these eggs.

Dr. Tyler: Have you tried a hyaluronidase preparation from bull testes or other sources?

Dr. Berg: No, I haven't.

Dr. Tyler: We have tested it quite extensively on sea urchin eggs without finding any definite effect.

Dr. Spikes: Can you get the same effect by putting eggs in calcium-free sea water?

Dr. Berg: Just recently I found if one takes off the membrane, then with the proper treatment with calcium-free sea water they will separate, which suggests again it is the matter of dissolving the cement between the cells.

Dr. Spikes: That would indicate at least two agents in the sperm extract, one for the membrane and one for the cement.

Dr. Berg: That was what I was trying to find out, whether there were really two substances there. The only way to answer that would be to try to carry out chemical studies of the sperm extracts.

Dr. A. R. Moore (University of Portland): I noticed there are two types of membrane, the fertilization membrane and the one which clings closely along the blastomeres. Those can't be the same.

Dr. Berg: The photographs don't show the membranes, and I believe what appeared to be a membrane was an artefact. Ordinarily it is very difficult to see the membrane as it is transparent and only one or two microns thick. You might be able to make out the edge.

Dr. Krauss: Dr. Berg, did you form any concept concerning the possible role of this substance in the fertilization of the egg?

Dr. Berg: No, I haven't. Of course, as Dr. Tyler has mentioned, the lysin may play a role in allowing the sperm to penetrate through the membrane.

Dr. Tyler: For that purpose it would not be necessary to dissolve the membrane completely. In the mammalian egg it has been shown recently that fertilization can be accomplished without any marked dispersal of the cumulus cells or dissolution of the cementing substance that holds them around the egg. This has inclined some investigators to the view that hyaluronidase is of no particular significance in penetration. I think it is clear, however, that a single spermatozoon can carry enough of the lytic agent to dissolve a path for itself, which may subsequently close over. There is no reason for assuming, at present, any joint action by a large number of sperm.

DR. CHAMBERS: This summer William White studied the action of purified beef testis hyaluronidase supplied by Dr. Duran-Reynals and sea urchin sperm extract prepared according to the method of A. Claude for the isolation of hyaluronidase. He found that the beef testis hyaluronidase had no effect on the extraneous coats. However, the sea urchin sperm extracts used in a dilution of 1 to 1,000 caused a swelling and dissolution of the jelly, but had no effect on the fertilization membrane and hyalin plasma layer.

DR. TYLER: Mr. Krauss and I have examined quite extensively the action of beef hyaluronidase on sea urchin eggs and our results agree with what you report were obtained by Dr. White. On the other hand, the dissolution of the gelatinous coat by a sperm extract, which was also reported last year by Monroy and Ruffo, we find to be interpretable on the basis of contraction of the precipitation membrane to the surface of the egg proper, as is illustrated in Fig. 5 of my paper.

DR. WILLIS PEQUEGNAT: I am interested in the effect on the blastomeres because the inhibitor I mentioned a while ago caused a similar effect in sea urchin eggs. Arbacia eggs were exposed to this substance for a considerable time and then fertilized. They were completely rounded up and sometimes there was complete separation. Also in relation to the corona effect you mentioned, essentially the same thing appeared in these preparations.

DR. BERG: There are other things, such as alkaline sea water, which will also cause these radiations.

DR. GIESE: Did you try the extracts of sperm in other forms?

DR. BERG: The only form I tried besides Mytilus was

Urechis and the results were completely negative. Also Mytilus sperm extract had no effect on Urechis eggs and *vice versa.*

DR. SCHECHTMAN: In Mytilus or other organisms that have been studied for the lytic effect, does the treatment of the egg with the lytic preparation result in a definite removal of the lytic principle? In other words, would eggs subsequently put into the solution be markedly protected?

DR. BERG: I haven't tried it. Perhaps Dr. Tyler has.

DR. TYLER: We have absorption experiments that show lowering of activity of the lytic agent. They are not good enough quantitatively to answer the question of whether or not the eggs can absorb it completely.

DR. SCHECHTMAN: I don't recall any one having found this. Perhaps you have worked on it, but I don't recall. Has any one found that the injection of sperm produces an anti-lytic substance which can remove all the activity?

DR. TYLER: We have made anti-sera against sperm extract containing the lytic agent of Megathura. In fact, anti-sera against inactivated (by heat) extract will inactivate the lytic agent.

CHAIRMAN ENGLE: In place of Dr. Gilbert Smith, who was unable to attend this conference, Dr. Markert, of California Institute of Technology, has agreed to report briefly on a diffusible sex-stimulating substance in a fungus. We shall now hear from Dr. Markert.

SEXUALITY IN THE FUNGUS, GLOMERELLA[1]

DR. CLEMENT L. MARKERT
CALIFORNIA INSTITUTE OF TECHNOLOGY

DR. SMITH was scheduled to speak this morning on his studies of the sexual substances of the unicellular alga, *Chlamydomonas,* but since he wasn't able to be here, I should like to say a few words about sexual substances in another plant—the fungus, *Glomerella.* This fungus is a common plant pathogen which produces one of the diseases known as anthracnose. The phenomenon of sexuality in *Glomerella* has been studied by Werner Hüttig (1935) in Germany and by several investigators at Louisiana State University, notably Lucas *et al.* (1944), Edgerton *et al.* (1945), Chilton *et al.* (1945), Lucas (1946), Wheeler *et al.* (1948).

Glomerella exists in a number of different strains which can be distinguished by their different degrees of fertility in sexual crossing. The usual method of demonstrating the mating reaction in *Glomerella* involves the inoculation of a petri dish containing 2 per cent. cornmeal agar (Difco) with the various mating types. When the strains, which are placed at opposite sides of the petri dish, grow together in the center, sexual crossing occurs (see Figs. 1 and 2). This crossing reaction results in the formation of a row of black perithecia—which are spherical reproductive structures containing the ascospores. The significant fact, from the viewpoint of sexuality, is that different strains react with one another to different degrees—that is, they exhibit different degrees of fertility. Environmental factors such as temperature, moisture, nutrition, etc., also affect the number of perithecia which are formed at the line of juncture between two mating types. Nevertheless, when environmental conditions are controlled, the number of perithecia formed by any two strains is constant enough to be a reasonably good measure of their mutual fertility.

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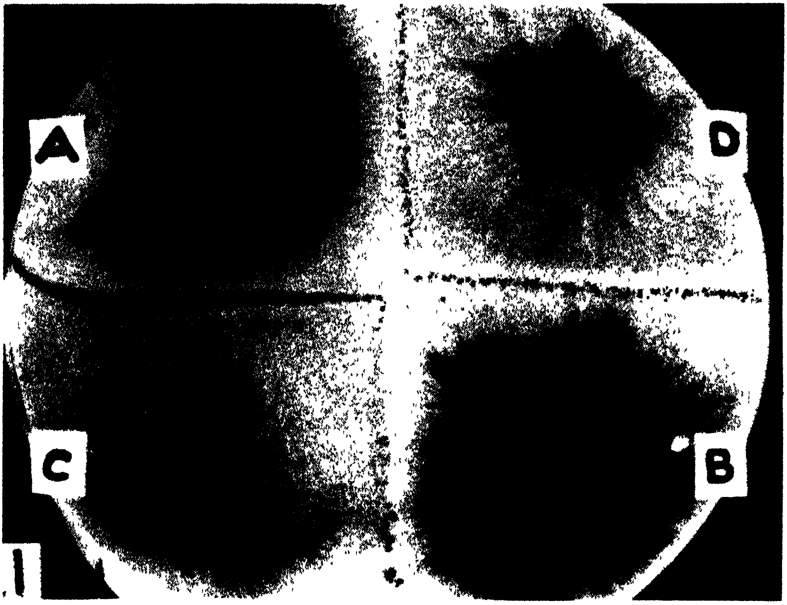
[1] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.

The strains of *Glomerella* may be divided into two basic groups: (1) those that produce perithecia homothallically, and (2) those that do not. In general, almost any two strains of *Glomerella* will cross with one another to some extent, provided that at least one of the strains carries the factor for perithecia formation. This brings me to the first general principle that I should like to mention—namely, the fertility of any cross depends upon complementary genetic factors in each strain. Thus, no one strain is more fertile than all the others in all possible crosses. For example, in comparing the fertility of strains *C* and *D*, we find that *C* is the more fertile in crosses with strain *A*, but that *D* is the more fertile in crosses with another strain *B* (see Fig. 1).

The second general principle which I want to bring out is that apparently the fertility of the cross between two strains is dependent upon some diffusible chemical substance. This principle was demonstrated in the following way: A petri dish was prepared with two layers of cornmeal agar separated by a cellophane membrane. Inoculated on the top layer of agar were strains *A* and *D* which normally cross weakly with one another. Underneath the cellophane membrane was inoculated strain *C*, which normally crosses very strongly with strain *A*. Strain *C* grew throughout the agar beneath the cellophane membrane but did not penetrate the membrane. When strains *A* and *D* finally met in the middle of the petri dish they crossed very strongly as shown by the formation of a dense row of perithecia. In fact, the cross between *A* and *D* seemed stronger under these circumstances than the normal cross between *A* and *C*, which is a very strong cross, indeed. It is apparent, there-



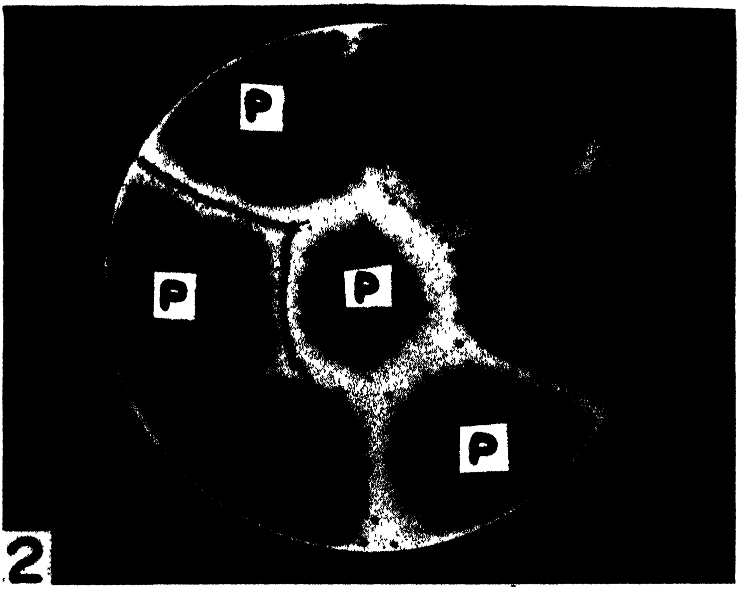


Fig. 1. The magnitude of the crossing reaction between strains A + C, B + D, A + D, and B + C is dependent upon the possession of complementary genetic constitutions by the crossing strains. Consequently, no single strain can be described as more fertile than all other strains.

Fig. 2. Examples of the crossing reaction between various strains of *Glomerella*. The strains labeled *P* (perithecial) form perithecia homothallically and also by sexual crossing with adjacent strains.

fore, that some substance produced by strain *C* diffuses through the cellophane membrane and stimulates the crossing reaction between strains *A* and *D*.

In summary, then, the two important principles illustrated by these experiments on *Glomerella* are, first, that the degree of fertility between the various strains of this organism is controlled by complementary genetic factors, and second, that these genetic factors exert their effects in part, at least, by means of diffusible substances. These same principles may, of course, apply also to higher organisms.

LITERATURE CITED

Chilton, S. J. P., Lucas, G. B., and Edgerton, C. W.
1945. *Am. Jour. Bot.*, 32: 549–554.
Edgerton, C. W., Chilton, S. J. P., and Lucas, G. B.
1945. *Am. Jour. Bot.*, 32: 115–118.
Huttig, Werner
1935. *Biol. Zentralbl.*, 55: 74–83.
Lucas, G. B., Chilton, S. J. P., and C. W. Edgerton
1944. *Am. Jour. Bot.*, 31: 233–239.
Lucas, G. B.
1946. *Am. Jour. Bot.*, 33: 802–806.
Wheeler, H. E., Olive, L. S., Ernest, C. T., and Edgerton, C. W.
1948. *Am. Jour. Bot.*, 35: 722–728.

DISCUSSION

CHAIRMAN ENGLE: That is a very interesting point. You are continuing with these experiments, I take it.

DR. MARKERT: Yes, these are only preliminary experiments. Further experimentation may of course modify these conclusions somewhat.

DR. TYLER: You avoided using the term "relative sexuality."

DR. MARKERT: I do not believe the concept of relative sexuality is applicable to *Glomerella* because no single strain is uniformly more fertile than all the others in all crossing combinations. Furthermore, the strains of *Glomerella* can not really be divided into two sexes, since in general almost all strains will cross with all other strains, including itself, provided that at least one of the strains carries the factor for perithecia formation. Hartmann's concept of relative sexuality was applied to *Glomerella* by Hüttig (1935). However, Hüttig's use of this concept was probably based upon the study of an insufficient number of strains of *Glomerella*. A few strains can be selected which do fit the concept of relative sexuality; but when all strains are considered some other concept becomes necessary to explain their sexual behavior.

DR. SCHECHTMAN: I wondered if you had tried, let us say, *A* and *C* on top and *D* below. In other words, would there be evidence of inhibiting diffusible substances?

DR. MARKERT: I tried the particular combination you mentioned. There was no inhibition. However, I haven't tried all possible combinations, and some strains may yet be found which do exert an inhibiting effect upon the crossing reaction between other strains. Whether there are many strains which can promote the crossing reaction between other strains, I do not know.

THE RELATION OF IONS TO THE APPEARANCE AND PERSISTENCE OF THE FERTILIZATION AND HYALINE MEMBRANES IN THE EGGS OF THE SEA URCHIN[1]

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THE elevation of the fertilization membrane is the first clear evidence of fertilization in the echinoderm egg. As soon as this membrane has reached its full extension, the hyaline layer appears on the surface of the egg and continues thickening, reaching its full form, 2.4 micra thick, in 15 to 20 minutes at 20° C. These two structures surround the dividing egg, the fertilization membrane serving as a cushion for the early embryo, providing a milieu of comparative constancy, while the hyaline layer holds the blastomeres together so that, as a result of their divisions, they form a hollow sphere.

The following discussion concerns the questions of the origin and nature of the fertilization membrane and of the hyaline layer. In order to throw light on these matters it may be helpful to consider an experiment published some years ago which showed that if the unfertilized eggs are first put into an isosmotic solution of urea or other non-electrolyte and, after a minute or longer, are removed to sea water, they can be fertilized, but no membranes form (Moore, 1930). Segmentation occurs in normal fashion, the cell-divisions taking place after the same time intervals as in

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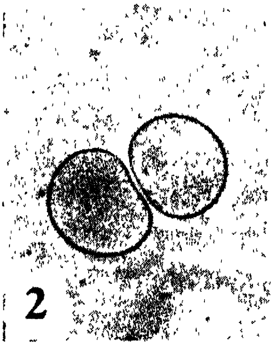
[1] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.



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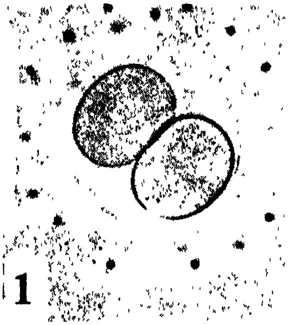

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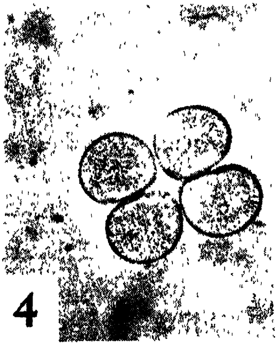

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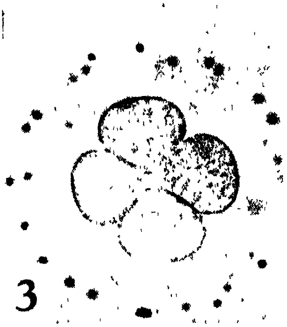

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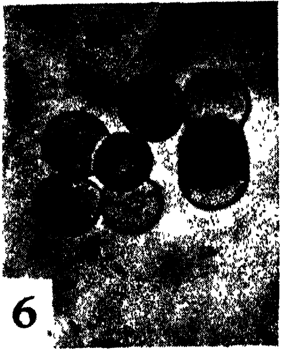

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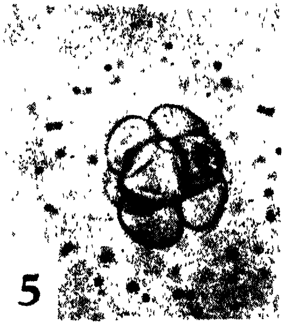

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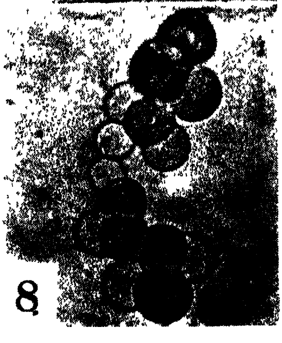

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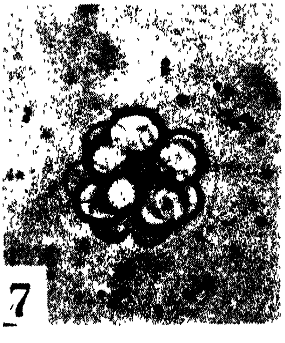

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the untreated controls (Figs. 1–8). This is sufficient to show that criticism of the experiment to the effect that cleavage is abnormal in these membrane-free eggs (Moser, 1940) is unfounded in fact. The only abnormality the embryos exhibit is the direct consequence of the lack of membranes, as a result of which the blastomeres form plates or cups instead of closed blastulae.

As to why eggs which have been immersed in a solution of non-electrolyte fail to form membranes after having been returned to sea water and fertilized, I have suggested that the pre-membrane material is dissolved out by any solution which lacks cations in a certain concentration and of which the pH is greater than 5. The facts which support this view have been made clear in a series of experiments with the eggs of a number of species of sea urchins (Moore, 1935a) in which it has been shown that the preservation of the membrane-forming function of the egg depends upon the pH and the concentration of the metal ions in the solution surrounding the eggs. For example, in a solution of glycerol it was found that the effectiveness of the solution in destroying the pre-membrane system fell off only slightly as the pH varied from 9 to 5, but that between 5 and 4 it fell to zero (Moore, 1932a) (Fig. 8a). Thus the membrane-forming capacity of the egg is not destroyed in the absence of metal ions if the pH is 4 or lower. It must therefore be concluded that the active agent which prevents membrane formation, *i.e.*, destroys the pre-membrane stuff, is the hydroxyl ion.

It can be shown that the cations of inorganic salts antagonize this destructive action of the hydroxyl ion, for if a given quantity of inorganic salt be added to the solution of non-electrolyte, it protects the membrane-forming capacity of the eggs even in alkaline solutions. The series (Table 1)

FIGS. 1, 3, 5, 7 show normal cleavage in the egg of *Dendraster excentricus*. The first four divisions. FIGS. 2, 4, 6, 8 show cleavage of a membrane-free egg from the same female. The times of division are exactly the same as in the normal. Note that, except for the membranes, the blastomeres are entirely normal in appearance.

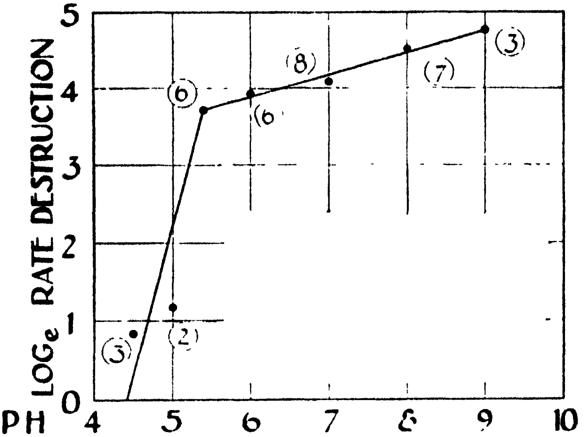


FIG. 8a. Showing the effect of pH on the rate at which the membrane-forming function of sea-urchin eggs is destroyed in an isosmotic solution of glycerol. Rate $= \frac{1000}{\text{time in seconds}}$. The numerals in small circles denote the number of observations from the average of which is derived the adjacent point on the graph.

shows that while a given minimum concentration of the salt varies among the species, it is constant for a given species. Thus, in the eggs of Temnopleurus the concentration for the chlorides of the lithium series is approximately 0.235 M,

TABLE 1

EACH NUMBER DENOTES THE MOLECULAR CONCENTRATION OF THE CORRESPONDING SALT, GIVEN IN THE LEFT-HAND COLUMN, WHICH IS JUST SUFFICIENT IN A SOLUTION OF UREA M/1 AT pH 8, TO PROTECT THE MEMBRANE-FORMING CAPACITY OF 50 PER CENT. OF THE EGGS. Co DENOTES COBALTAMINE CHLORIDE AND THE NUMBER AFTER IT THE VALENCE. THE MONOVALENT COBALTAMINE CHLORIDE, SINCE IT WAS WITHOUT EFFECT, IS OMITTED FROM THE TABLE.

	Clypeaster japonicus	Temnopleurus hardwickii	Pseudocentrotus depressus	Strongylocentrotus pulcherrimus	S. nudus	Salt avg.	Average effectiveness numbers
LiCl		0.21	0.10	0.17	0.24	0.18	5.5
NaCl	0.25	0.21	0.10	0.20	0.22	0.19	5.3
KCl		0.28	0.10	0.13	0.25	0.19	5.3
RbCl		0.22	0.12	0.17	0.25	0.19	5.3
CsCl		0.25	0.12	0.17	0.25	0.20	5.0
Species average	0.25	0.235	0.11	0.17	0.24		
$MgCl_2$	0.0074	0.0068	0.0038	0.0045	0.0069	0.0059	169.5
$CaCl_2$	0.0060	0.0042	0.0023	0.0037	0.0028	0.0038	263.1
$SrCl_2$	0.0064	0.0068	0.0038	0.0030	0.0046	0.0049	204.0
$BaCl_2$	0.0042	0.0046	0.0023	0.0020	0.0036	0.0034	293.8
Co_2	0.0025	0.0020	0.0015	0.0020	0.0020	0.0020	500.0
Co 3	0.00025	0.00004	0.0002	0.0001			
Co 4	0.00020			0.0001			
Co 6	0.00005			0.00002			

but in Pseudocentrotus it is 0.11 M and in *Strongylocentrotus nudus* it is 0.24 M and very nearly the same for *S. purpuratus*. From these facts it must be concluded that the protective effect of the monovalent series is a function of the valence of the cation, and that there is no specific effect of a particular cation. But with the divalent series of metals another factor appears in that there are differences between specific cations. Thus while Ca is 50 to 100 times as potent in its action as Na, the ion Mg is always much less effective than Ca; while Sr and Ba approximate Ca in their effectiveness. It is clear that something other than valence plays a part, some specific property of the divalent ion. It should also be noted that unlike many other physiological reactions there is no evidence of antagonism between mono- and di-valent cations; here their action is synergic (Moore, 1932b). In this there is a striking parallelism between the effects described for sea urchin eggs and those discovered by Gray (1931) for the solubility of the intercellular matrix of the ciliated epithelium of the gills of Mytilus. Gray sums up the matter thus: "The real dispersive agent is the hydroxyl ion since this ionizes the matrix. All salts inhibit the process, divalent ions being much more powerful than the monovalent cations."

However, the effect of increasing the valence is so striking in the case of the cations, that it seemed important to determine whether the valence of the anion also might possibly be a factor. During the past season a series of experiments has been carried out by R. S. Dean and myself for the purpose of testing the matter (Table 2). The question received a clear answer, which is indicated by the two salts of Mg,

TABLE 2

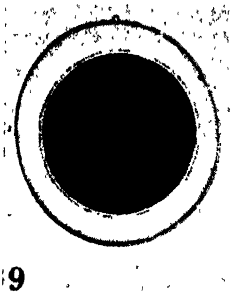
THE CONCENTRATIONS OF SALTS EFFECTIVE IN PROTECTING THE MEMBRANE-FORMING CAPACITY OF THE EGGS OF *Strongylocentrotus purpuratus*

Li Cl	0.2 M	Na_2 oxalate	0.12 M
Na Cl	0.2 M	Na_2HPO_4	0.11 M
KCl	0.2 M	Na_2SO_4	0.10 M
		Na_2 tartrate	0.10 M
$MgCl_2$	0.017 M	Na_3 citrate	0.07 M
$CaCl_2$	0.004 M	$K_4Fe(CN)_6$	0.10 M
$SrCl_2$	0.007 M		
$MgSO_4$	0.017 M		

the chloride and the sulfate, in that their effective concentrations are identical. The difference in the valence of the anions has no effect on the minimum concentrations. Na_2SO_4 is twice as effective as the chloride of the same molecular concentration, and the Na_3 citrate is three times as effective, results to be expected if the dissociation of the salts is complete, indicating that the effect depends on the normality of the solution. $K_4Fe(CN)_6$ is, however, only twice as effective as the chloride of the same molecular concentration. These results demonstrate that multiple valences of the anions are without effect on the reaction; only in the case of the cations is valence important. When it is remembered that the tetravalent cobaltamine chloride is 1000 times as effective as the monovalent cation, the failure of the 4-valent anion $Fe(CN)_6$ to affect the reaction is evidence as clear-cut as it could well be that the valence of the anion is of no moment in this reaction.

In considering these quantitative aspects of the protective action of the cation we left aside for the moment the question of how it is that in so brief a time as 30 seconds a solution of urea, for example, can render the egg incapable of forming a membrane. That the initial action of the urea solution is extremely rapid is shown by Motomura's (1934) experiment in which the eggs are exposed to a solution of non-electrolyte for 6 or 7 seconds, then returned to sea water in which the normal fertilization membranes soon appear, an instance of artificial parthenogenesis (Fig. 9). As in some other cases of artificial parthenogenesis, the membrane appears, not in the activating solution, but after the eggs are returned to sea water. In recent experiments with the eggs of *S. purpuratus* I have found that the action of the urea solution is extremely rapid, less than 2 seconds' immersion is necessary at 2° and 20° C. to activate the egg. This indicates a temperature coefficient near unity and suggests that a physical process, such as solubility, constitutes a sort of trigger action for initiating the formation of the membrane. The other possibility is that the time required for the reaction is a fraction of a second. This would necessitate the observation of a temperature-differential of rate in less than the shortest time necessary for an observation, and this difficulty has so far not been surmounted.

The role of the ions in forming the fertilization membrane and maintaining its integrity is made clear by an



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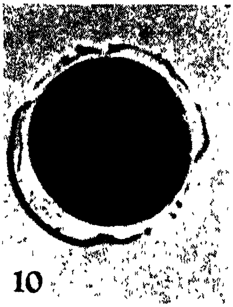

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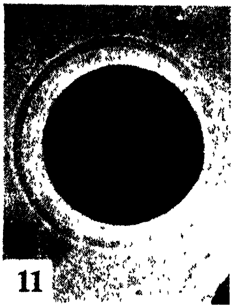

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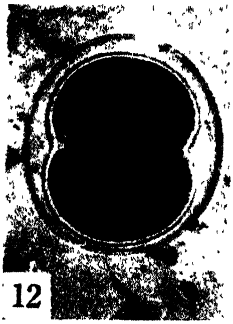

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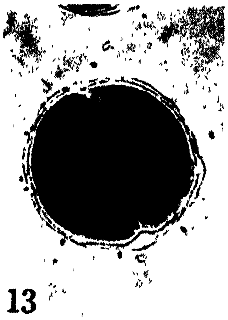

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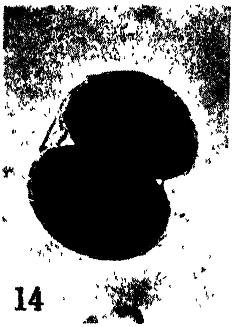

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FIG. 9. Normal fertilization membrane and hyaline layer formed by Motomura's method in *Strongylocentrotus purpuratus*. FIG. 10. Same but with fertilization membrane crumpled by the addition of 10 drops of 1 per cent. gelatine to the sea water in which the egg lies. FIG. 11. Promembrane called forth in a solution of urea containing gelatine. This structure, unlike the fertilization membrane, is not crumpled by protein in the sea water about it. FIG. 12. Egg of *S. purpuratus* which has been exposed to a solution of M urea containing NaCl in 0.25 M concentration, and then fertilized. 2-cell stage, perfectly normal. FIG. 13. Same, but with the urea solution containing 0.12 M NaCl. The fertilization membrane has been formed following fertilization but not elevated. FIG. 14. Same, but with the urea solution containing 0.06 M NaCl. The fertilization membrane is absent, but the hyaline layer is complete.

experiment in which the eggs are transferred to a urea solution at intervals after being fertilized in sea water. If such eggs are put into the urea bath 30 seconds after fertilization, both fertilization and hyaline membranes fail and the eggs proceed to divide into blastomeres which form chains; after 45 seconds, fertilization membranes are present but fragile and much distended, quite abnormal. Even after 3 minutes, when the membrane is fully formed and elevated, immersion in the ion-free solution causes the structure to be weakened and distended. It is only when the membrane is 4 or 5 minutes old that it is able to resist the dissolving effects of a salt-free solution.

On the question of the origin of the fertilization membrane, there are two hypotheses, namely, either the membrane is formed by the interaction of pre-membrane substances in the cortex following the entrance of the sperm, or is already formed on the surface of the unfertilized egg, activation serving only to lift it. That the fertilization membrane is a structure strikingly different from the membrane on the unfertilized egg is shown by the following facts obtained from experiments on the eggs of *S. purpuratus:*

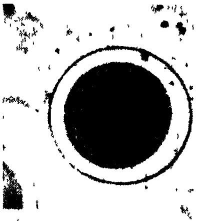
(1) The fertilization membrane at first is fragile and elastic, dissolving in an ion-free solution. In sea water, however, the membrane develops, during the first 4 or 5 minutes, its characteristic toughness and rigidity. One element, therefore, in the maturation of the membrane consists in the salts of sea water. E. N. Harvey has suggested that oxidation may play a part as it does in the hardening of the silk fiber, on exposure to air, after it leaves the body of the worm.

(2) If it is assumed with Runnström that before fertilization the membrane lies on the egg as a curved sheet consisting of folded protein chains, and that the effect of fertilization is an unfolding, and hence enlargement of the structure, we should expect that, when osmotic pressure is applied by means of large protein molecules in the surrounding solution (Dean and Moore, 1947), the resulting shrinking of the membrane would take place in a regular

pattern of wrinkling. This does not occur, but instead the crumpling is entirely irregular, giving no indication of a previously existing wrinkle pattern (Fig. 10).

If we take the view rendered probable by these considerations, namely, that the fertilization membrane exists, not as a formed membrane on the unfertilized egg, but as a gen or precursor in the cortex of the egg, then we must suppose that the precursor substance or substances leave the egg during the 60 seconds or less that the egg remains in the solution of non-electrolyte, as described in the foregoing experiments. The failure of the fertilization membrane to appear on eggs treated in this way when they are returned to sea water and fertilized, as stated above, has been ascribed to the action of the hydroxyl ions in dissolving out the premembrane material from the cortex of the egg (Moore, 1935b). In the earlier experiments the loss of substance from the egg escaped visual detection. I have found, however, that if a little protein, gelatin or albumen be added to the salt-free solution, the loss of material from the egg becomes at once apparent. This pre-membrane stuff appears in two stages. The first, which we may call pro-membrane A, is seen as a clear membrane pushing rapidly out of the egg in the first few seconds after immersion. The second, pro-membrane B, follows in approximately 10 seconds, and appears as a denser, more refractive zone within the first (Fig. 11). The boundary of B never overtakes that of A. Both extend enormously, far beyond the limits of a normal fertilization membrane (Figs. 16, 17). This fact of the extensibility of the pro-membrane emphasizes the remarkable property of the fertilization membrane in its precisely limited extension, when completely formed. The protein nature of A and B is shown by the fact that they quickly disappear in a solution of urea containing trypsin. This establishes a point of identity as to chemical constitution between promembranes and newly formed fertilization membranes which also dissolve in sea water containing trypsin.

A short time after the eggs which have been bathed in a solution of non-electrolyte are immersed in sea water and the pro-membranes dissolved, a third substance, a clear granular mass exudes from the egg (Fig. 18). Its timing and reactions indicate that this substance may be considered the stuff which, under normal circumstances, makes up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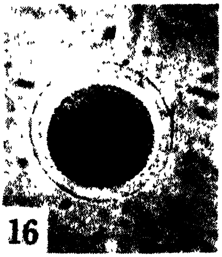
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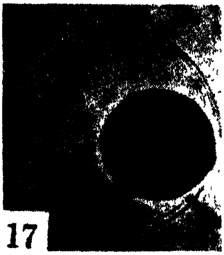
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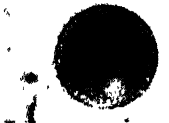
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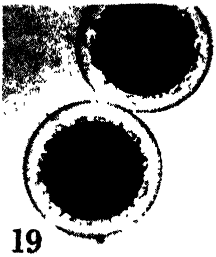
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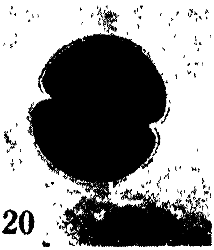
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FIG. 15. Normal fertilization membrane in an egg of *S. purpuratus*. FIG. 16. Beginning of promembranes A and B 10 seconds after putting the egg into 10 cc. of solution of urea M/1 + 0.5 cc. 1 per cent. gelatine. FIG. 17. Same 30 minutes later. Note extension and the fact that the two protomembranes do not coalesce. FIG 18. The egg was returned to sea water after the formation of protomembranes A and B. These quickly dissolve and in a few minutes clear granules come out of the egg, forming a mass which reacts in acid water and in a solution of non-electrolyte, exactly like the hyaline layer. Hence the exudate may provisionally be called prohyaline substance. FIG. 19. Fertilized eggs of *S. purpuratus* put into sea water which has been rendered acid to pH 3.5. Here the hyaline layer loses its structure and forms droplets. This layer behaves as if it were a calcium proteinate. FIG. 20. Egg which has been put into trypsin sea water 2 minutes after fertilization. The newly forming fertilization membrane has been dissolved by the trypsin, but the hyaline layer remains intact in the trypsin sea water solution.

the hyaline layer, for it is soluble in acid sea water (Fig. 19), in M/2 NaCl and in salt-free solutions. It thus acts in all ways like the hyaline layer of the developing egg and may be referred to as the pro-hyaline substance.

We are now in possession of sufficient facts to give a provisional account of the loss of materials by eggs subjected to treatment with a solution of non-electrolyte. In this bath the unfertilized egg excretes the precursor substances necessary for building the fertilization membrane. These substances are the pro-membranes A and B, which, when the eggs are immersed in sea water, quickly dissolve, and the stuff which, in the normal egg, constitutes the hyaline membrane, exudes as an unformed mass. As a consequence, after fertilization, eggs which have been treated in this way develop without supporting membranes. The blastomeres are held together only by cell connectives, and form irregular clusters or plates.

Further information on the nature of pro-membranes A and B may be obtained in another way, namely, by trypsin digestion before fertilization.[2] In order to demonstrate this effect, the unfertilized eggs are put into a solution of trypsin in sea water (100 mg. per cent.) for a certain length of time, and are then transferred to sea water and fertilized. In those which have remained 30 seconds in the trypsin bath before fertilization, normal membranes may develop; on those removed from the bath after 60 seconds, membranes are formed, but not elevated, with the result that the embryos are nearly solid blastulae; after 2 minutes in the trypsin bath no membranes are formed, the blastomeres being held together by the hyaline layer (*cf.* Figs. 12, 13, 14). The experiment shows that the premembrane material in the cortex is dissolved by the trypsin within 2 minutes, and that the normal elevation of the membrane depends upon the presence, between the membrane and the egg, of protein molecules which are dissolved before those which go to form the membrane. It should be noted that, like the action of ion-deficient solution on the premembrane system, the digestion of the precursors in the egg takes place by steps.

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[2] In a personal communication, September, 1932, Dr. Kunitz told me of experiments with trypsin which he had carried out on eggs of Arbacia. From the results he concluded that "trypsin has an effect on the precursor of the fertilization membrane, but does not affect the membrane once it has (fully) formed."

Another method of studying the chemical constitution of the membrane is to submit the fertilized eggs to a trypsin bath after they have been fertilized. If put into this bath 10 seconds after fertilization they subsequently form, in sea water, chains of blastomeres; put into the trypsin bath 2 or 3 minutes after fertilization, the fertilization membrane is dissolved, but the hyaline membrane remains unaffected, holding the blastomeres together to form a blastula. This development may take place even if the eggs remain in the trypsin bath. The fertilization membrane thus readily undergoes the digestion characteristic of proteins, while the digestion of the hyaline layer takes place slowly if at all. Runnström (Runnström, Monné and Broman, 1944) reports that he met with no difficulty in dissolving the hyaline layer with trypsin. This is not unqualifiedly true in my experiments for all concentrations of trypsin. Even in eggs divested of the fertilization membranes, lying in the trypsin bath, the hyaline layer persists (Fig. 20). Thus the newly formed fertilization membrane is more soluble in trypsin than is the hyaline layer. However, if the trypsin solution is used in higher concentration, say 400 mg. per cent., the hyaline layer also is dissolved. It is interesting to compare the results of trypsin digestion in different places with different forms. Kunitz and later Chambers and White (1949) found that in Arbacia the precursor is dissolved by trypsin. This is identical to Runnström's report for two different species on the Swedish coast and to my results with *S. purpuratus*. Therefore, with respect to the trypsin solubility of precursor system, all agree. But in disagreement with Runnström, I have found the fertilization membrane, often up to 5 minutes after fertilization and in one lot of eggs up to 30 minutes, is dissolved by trypsin. The fertilization membrane is therefore protein in its constitution.

That the fertilization membrane differs unmistakably from its precursors is made clear by the following table of properties:

FERTILIZATION MEMBRANES	PRO-MEMBRANES A AND B
1. Early solubility in ion-free solution.	1. Insoluble in ion-free solution.
2. Insoluble in sea water.	2. Soluble in sea water.
3. Rigid when set.	3. Not rigid.
4. Collapsed by small amount of protein.	4. Not collapsed by small amount of protein.
5. Dissolved by trypsin in early stages.	5. Dissolved by trypsin in all stages.

CONCLUSIONS

The unfertilized egg contains in its cortex two substances, pro-membranes A and B which, if released simultaneously, react to form the fertilization membrane and to provide for its elevation. This release suggests a trigger reaction, normally set off by the entrance of the sperm, but which may be tripped by any one of the numerous parthenogenetic agents. The reaction can be completed only in the presence of salts, *i.e.*, *cations*. In the case of exposure of the unfertilized egg to non-electrolyte solution, A is followed by B after an interval. As these structures extend, the boundary of B approaches that of A but never overtakes it. At least two factors thus seem to be necessary for the formation of the fertilization membrane:

(1) The very nearly simultaneous release of A and B.

(2) The presence of salts. The facts of Motomura's experiment suggest that salts are essential in forming the membrane.

It is suggested that the origin of A and B may be the granules of the cortex described by Moser (1939). The difference in time of appearance in the case of the two precursors may be referred either to a difference in the rates of solubility or to a spatial difference in the distribution of the substances, *i.e.*, the granules destined to form B may be deeper lying. The result in either case would be temporal and spatial differences in the appearance of A and B which would mean that there must be at least two types of granules, with possibly a third to account for the pro-hyaline material, and a fourth to provide the large molecules for the elevation of the membrane by osmotic pressure.

The relation of A and B and salts to the formation of the membrane suggests that the mechanism may be analogous to the clotting of blood or milk. In the latter two systems the precursors exist side by side without interaction until a mobilizing factor by a sort of trigger action changes the precursors into reactive forms and the result is a water-insoluble clot. In the egg the fertilization membrane is the final result.

LITERATURE CITED

Chambers, Edward, and William White
1949. Personal communication.
Dean, R. B , and A. R. Moore
1947. *Biodynamica,* 6: 159–163.
Gray, J.
1931. "Experimental Cytology." Cambridge University. Pp. 516, p. 112.
Moore, A. R.
1930. *Protoplasma,* 9: 9–17.
1932a. *Jour. Cell. Comp. Physiol.,* 2: 41–51.
1932b. *Protoplasma,* 15: 268–275.
1935a. *Jour. Cell. Comp. Physiol.,* 5: 433–440.
1935b. *Scientia,* pp. 92–100.
Moser, F.
1939. *Jour. Exp. Zool.,* 80: 447–464.
1940. *Biol. Bull.,* 78: 68–79.
Motomura, I.
1934. Science Reports of Tohoko Imperial University. 4th series, 9: 33.
Runnstrom, J., L. Monné and L. Broman
1944. *Arkiv for Zool ,* 35A (3): 1–32.

DISCUSSION

CHAIRMAN ENGLE: Thank you, Dr. Moore. We shan't be able to give the time for discussion that it deserves, and due to that lack of time will the discussants make their comments and questions short.

DR. CHAMBERS: I was wondering whether in Strongylocentrotus there is a very specific action on the membrane relative to monovalent ions. By putting eggs into the salt shortly after fertilization the membrane is actually formed. You can't see it, but you can demonstrate it with a needle. As soon as you add strontium it becomes a definite structure. Do you think calcium and strontium could combine with it in a more physical manner than the sodium?

DR. MOORE: That might serve to account for this great difference between calcium and strontium on the one hand and the other salts. I think that your suggestion is a reasonable one.

CHAIRMAN ENGLE: Are there any other comments?

DR. CHAMBERS: I would like to say something about the effect of urea. If we put Lytechinus eggs into urea and then into sea water, the nucleus moves to the center and starts to divide. We tried to see what ions are effective in bringing this about. Sodium was most effective. If we put them into isosmotic solutions of magnesium iodide, this result was not obtained.

DR. MOORE: It is possible your potassium-calcium ratio in the concentrations that are present in sea water could be the cause. Could you reverse the procedure? Could you take them out of the pure salt solution and put them into sea water and fertilize them?

DR. CHAMBERS: I never tried that.

DR. MOORE: It should be pointed out that sodium is the least toxic of the salts of sea water, whereas potassium is very toxic. This fact might account for the effect you describe.

DR. TYLER: Costello has recently described, in eggs of Nereis, an interesting separation of the membrane into two parts. When the eggs are placed in alkaline sodium chloride around pH 10, the membrane swells up, or probably the material under the membrane swells, and then, later the membrane splits into two parts.

DR. MOORE: That is apparently a later separation and a different phenomenon. The separation I have described is most marked at the start, and diminished later.

THE PHOSPHORUS COMPOUNDS OF SEA URCHIN EGGS AND THE UPTAKE OF RADIO-PHOSPHATE UPON FERTILIZATION[1, 2]

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EXTENSIVE work with invertebrate embryos, reviewed by Tyler (1942), and more recently with amphibian larvae by Barth and Jaeger (1947) and Hall and Moog (1948), has clearly shown that many facets of growth and differentiation are closely dependent on energy derived from respiratory metabolism. One of the primary problems of developmental physiology which grows out of these findings is to determine how energy is mobilized from substrates and transferred for use by the specific developmental process. The problem is analogous to that of energy transfer in muscle contraction, and, as in this instance, it is possible that phosphorylation and production of high-energy phosphate will play a central part. In a less direct manner, phosphorus must have an important role in genetic and cytoplasmic control of cellular processes in the embryo, because of its presence in desoxyribonucleic and ribonucleic acids, and recently evidence has been presented that involves certain phosphorylated compounds in control of

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[1] The author's contributions described in this article were made in 1946–1947 during the tenureship of a National Research Council Fellowship in Zoology at California Institute of Technology in the laboratories of Dr. Albert Tyler.

[2] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.

TABLE 1

PHOSPHORUS COMPOUNDS IN UNFERTILIZED ECHINODERM EGGS

(The data in this table have been recalculated, and in some instances the compounds identified more specifically than by the original investigators)

Phosphorus fraction	*Patiria miniata*	*Dendraster excentricus*	*Paracentrotus lividus*	*Brissopsis*	*Arbacia punctulata*	*Arbacia punctulata*	*Strongylocentrotus purpuratus*	*Lytechinus pictus*
Total P	6.64 mg./gm. dry weight	6.94 mg./gm. dry weight	134.7 mg./gm. N	. . . .	130 mg./gm. N 3.1 mg./gm. wet weight[4]	960 $\mu g/10^6$ eggs[1] 4.13 mg./gm. wet weight[2]	817 $\mu g/10^6$ eggs 2.94 mg./gm. wet weight	2.78 mg./gm. wet weight
Acid Insoluble P	*48%*	*50%*	*53.0%*	....	*62.4%*	*62.2%*	*69.1%*	*56.8%* [3]
RNA P	16%	32%	30.5%	. ... . .	4.6%	22.2%	38.0%	24.7%
DNA P				. .. . .	1.1%	0.9%	1.7%	0.5%
Phosphoprotein P	trace	12%		.... ..	19.1%	2.6%	2.4%	1.8%
Phospholipid P	32%	6%	22.5%		37.6%	36.5%	27.0%	20.5%
Acid Soluble P	*52%*	*47%*	*47.0%*		*37.6%*	*37.8%*	*30.3%*	*38.2%*
Inorganic P	20%	29%	28.7%	53% of ASP.	11.2%	. .	7.4%	15.1%
ATP + ADP P	13%	14%	11.4%	31% " "	21.3%	.	8.2%	13.8%
Propanediol phosphate P	19%	4%	7.6%	16% " "		.	12.0%	7.3%
Unidentified P				. . ..	5.1%	.	2.7%	2.0%
Investigator	Needham and Needham (1930)		Zielinski (1939)	Lindberg (1943)	Crane (1947)	Schmidt *et al.* (1948)	Whiteley	Whiteley

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[1] Schmidt *et al.* reported 96×10^{-3} μg/egg, but it is believed this should have read 96×10^{-5} μg/egg.

[2] Calculated from the per-egg basis using conversion data from Harvey (1932).

[3] 9.8 per cent. lost in fractionating acid insoluble components.

[4] Crane reports "0.31 milligrams per cent of wet weight."

morphogenesis in the echinoderms. It is the intent of this paper to review the compounds of phosphorus that have been identified in echinoderm eggs from the point of view of these three major uses.

Since the first relatively complete study of the phosphorylated compounds of echinoderm eggs by Needham and Needham (1930), analyses of various species have been published by Zielinski (1939), Lindberg (1943), Crane (1947), and Schmidt, Hecht and Thannhauser (1948). The data are gathered together in Table 1, along with figures obtained for the unfertilized eggs of *Strongylocentrotus purpuratus* and *Lytechinus pictus* by the author. There is relatively good agreement in the kinds and quantities of substances present despite differences in technique and species of sea urchin. Further refinement, and possible identification of additional compounds, will probably depend on development of more specific and accurate methods, such as the enzymatic methods of Kalckar (1947) for the phosphorylated adenosine compounds.

It is interesting that the greatest bulk of phosphorus is contained in ribonucleoprotein and phospholipids, while, in most instances, comparatively little phosphoprotein exists. The echinoderm egg is quite different, in these respects, from amphibian and chicken eggs which contain comparatively little ribonucleoprotein and a large amount of phosphoprotein (Needham, 1942). To date, only three acid-soluble phosphates have been isolated from the eggs of sea urchins: inorganic orthophosphate, propanediol phosphate, and adenosine triphosphate. In addition, three coenzymes have been measured manometrically in the unfertilized eggs of *Arbacia punctulata.* Diphosphothiamine is reported present to the extent of 2.3 micrograms per gram wet weight, or 10.0 micrograms per gram dry weight by Krahl, Jandorf and Clowes (1942), a figure confirmed by Goldinger and Barron (1946) who have found 12.9 micrograms per gram dry weight. Diphosphopyridine nucleotide is present to the extent of 250–500 micrograms per gram wet weight in the same eggs, approximately 25–40 per cent. in the dihydro-form (Jandorf and Krahl, 1942), and Krahl, Keltch and Clowes (1940) find an average concentration of flavin dinucleotide of 9.8 micrograms per gram wet weight. The sum of the phosphorus in these compounds would, to a first approximation, amount to about 2 per cent. of the total egg phosphorus, and could account for the bulk of the unidentified acid soluble phosphorus of *S. purpuratus* and *L. pictus* eggs (Table 1).

It is noteworthy that neither phosphoarginine nor phosphocreatine have been found in mature eggs, though both are present in adult tissues and at least phosphoarginine has been identified in the ovaries of *Brissopsis* (Lindberg, 1943).

NUCLEIC ACIDS

The large content of ribonucleic acid in sea urchin eggs is a feature they have in common with a number of invertebrate eggs. Brachet has proposed that this acid serves as a sort of reservoir of material to be converted into desoxynucleic acid as development proceeds and the number of nuclei increases. This view is thoroughly reviewed in his recent book (1945). It has been challenged by Schmidt, Hecht and Thannhauser (1948) on the basis of their finding that ribonucleic acid content remains constant through pluteus formation in *A. punctulata* while desoxynucleic acid increases markedly. Recently Villee *et al.* (1948) have shown that the radiophosphate incorporated into the acid insoluble fraction in *Arbacia* embryos is found in large part in desoxynucleic acid, with very little appearing in the ribonucleic acid. Moreover, malononitrile and other inhibitors depress ribonucleic acid synthesis in these eggs more than desoxynucleic acid synthesis. All these observations remove the force of Brachet's suggestion and lead one to look for a function for this large amount of ribonucleic acid elsewhere than as a precursor of desoxynucleic acid. It is not unreasonable to suppose that this function is concerned with differentiation of new proteins in the embryo, in view of the arguments brought forth by Brachet (1945) and by Caspersson (1947) implicating ribonucleic acids in protein synthesis.

Phosphoprotein and phospholipids, the other components of the acid insoluble phosphorus, have received little special study and will not be mentioned further here.

INORGANIC PHOSPHORUS

Inorganic phosphorus is present in appreciable concentration in the unfertilized egg, and it would be reasonable to suppose that increased needs for phosphate for biosyntheses, energy metabolism, or spicule formation during development might be met by liberation of phosphate from phospholipids or other organic sources. It has now been established, however, that, in the presence of phosphate in the sea water, the developing embryo can augment its content of phosphorus by an increased permeability to the ion, beginning very shortly after fertilization. Although the first observations on the uptake of radioactive phosphate in *Arbacia* eggs made by Brooks (1943) showed only a slight and erratic penetration, and more recently Lindberg (1946) reports the failure of the ion to penetrate, newer studies by Brooks and Chambers (1948), since confirmed by a number of investigators, show that there is indeed a very rapid uptake of the labelled phosphate. Though both unfertilized and fertilized eggs absorb the phosphate and accumulate it over and above the concentration in the ambient sea water, the uptake rate in fertilized eggs is 130 to 160 times as great as for unfertilized.

An example of the time course of accumulation in developing eggs of *Lytechinus pictus* is given in Fig. 1, from Chambers and Whiteley (1949). In this experiment, the uptake of phosphorus by a small sample of eggs in a special thin-walled vessel was followed by continuous recording with a Geiger counter while sea water containing 0.00043 microcurie of radioactive phosphorus per ml. was flowed over the eggs. The very marked increase in rate of uptake caused by fertilization is well shown, beginning after a lag of 12 to 15 minutes. This lag period corresponds in time with the initial dramatic respiratory changes that are so well known for echinoid eggs, and it is apparent that there is no direct relation between the two events. Similarly,

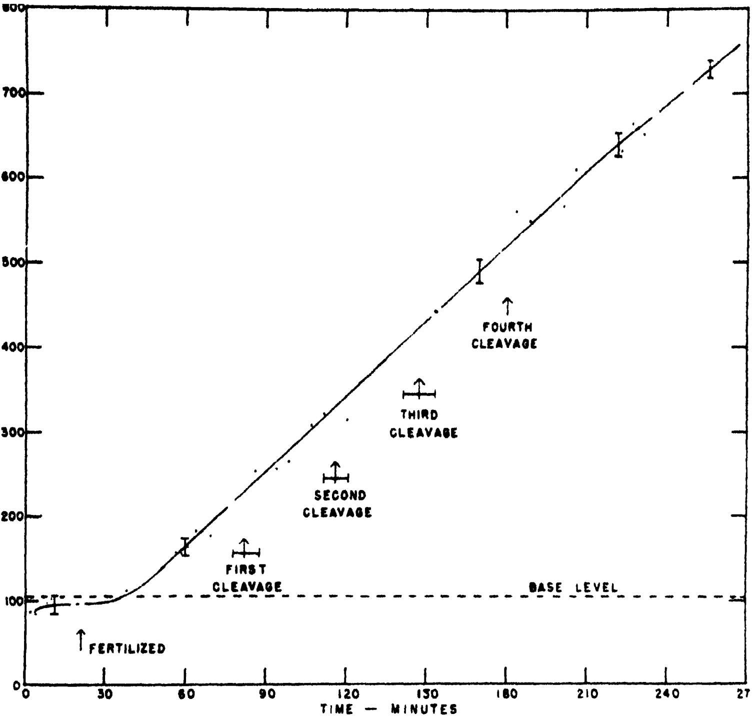

FIG. 1. P^{32} uptake by fertilized eggs of *Lytechinus pictus*. 0.00043 μC/ml. of P^{32} was flowed over the eggs at a rate of about 4 ml./min. at 24.5° to 25.5° C. until cooled at 170 minutes to 21° C. Radioactivity was measured continuously. The base level of activity of the system without eggs is shown by the horizontal line. The vertical bars on the curve indicate twice the standard error. Development of the eggs was 98 per cent. normal to the 128-cell stage.

there is no rhythmic pattern of phosphorus uptake during the mitotic phases, in contrast to the respiratory cycles known for urchin and other kinds of eggs (Zeuthen, 1949). The deviations from a smooth curve are all just within the limit of counting error. Attention was originally called to this lack of inter-relation between uptake and mitosis by Brooks and Chambers (1948).

The distribution of radiophosphorus in unfertilized eggs and in fertilized eggs and cleavage stages is almost entirely among the acid soluble phosphates, the acid insoluble fraction containing only 3.5 to 4.1 per cent. of the total, practically none of which is in the phospholipid material (Chambers, Whiteley, Chambers, and Brooks, 1948). The interesting questions of the distribution of the large acid soluble portion among the components of that fraction and the conditions determining turnover rates there deserve thorough study. Abelson (1948) has made a start in this direction for the eggs of *Arbacia*, finding variable amounts of the isotope in all components of the early embryos.

Though little difference exists between fertilized and unfertilized eggs as concerns the gross chemical distribution of the radiophosphorus, there is a pronounced difference in the cytological distribution. This was determined by bathing the eggs of *Lytechinus pictus* in sea water containing radiophosphorus and then centrifuging them into two parts by the centrifuge technique of Harvey (1941). Radioactivity determinations could then be made on the light fragments separated from the heavy ones. In *L. pictus*, the large, light parts contain an oil cap, a small hyaline layer, the nucleus or mitotic figure, and the yolk, while the smaller, heavier part contains mitochondria and a heavy, optically empty layer. Unfertilized eggs allowed to accumulate radiophosphorus for about 6 hours have the phosphorus distributed so that the lighter part receives 1 to 2.4 times as much as an equal volume of the heavier. Fertilized eggs, however, allowed to accumulate the isotope for 18 to 29 minutes and then centrifuged into two fragments have the isotope distributed between light and heavy fragments in the ratio of 0.48, 0.45 and 0.55 for three experiments. The bulk of the newly absorbed phosphorus in fertilized eggs is associated with mitochondria or the heavy hyaline material.

PROPANEDIOL PHOSPHATE

Propanediol phosphate is an ester first isolated and identified by Lindberg (1946) from cow brain. The identification of this ester in echinoderm eggs rests chiefly on the observations that the barium salt of propanediol phosphate is soluble in water and alcohol, which thus sets it apart from most of the heretofore known phosphate esters, and on the fact that it resists both mild acid and alkaline hydrolysis. At least in *S. purpuratus* this substance comprises the largest component of the acid-soluble phosphate. A hypothesis concerning the part played by this substance will be presented later.

ADENOSINETRIPHOSPHATE

The identification of adenosinetriphosphate is rather well established. The determinations of Table 1 are based on the familiar procedure of hydrolyzing the barium insoluble phosphate for 7 minutes at 100° C. in 1 N HCl. Recently, the ultraviolet absorption spectrum of the barium insoluble phosphate of *S. purpuratus* eggs has been measured by the author and is essentially the same as that of adenosinetriphosphate purified from rabbit muscle. These spectra, and that of adenosine for comparison, are shown in Fig. 2, curves *a*, *b* and *c*. When adenosine is deaminated by adenosine deaminase to form inosine, the absorption spectrum is changed as shown in *f* (Kalckar, 1944; Mitchell and McElroy, 1946a). In a like manner the spectra of the sea urchin and rabbit adenosinetriphosphates are changed by the deaminase to those of *d* and *e*. The enzyme was prepared by the procedure of Mitchell and McElroy (1946b). It is also reported by Chambers and White (1948) that they have observed that purified myosin causes hydrolysis

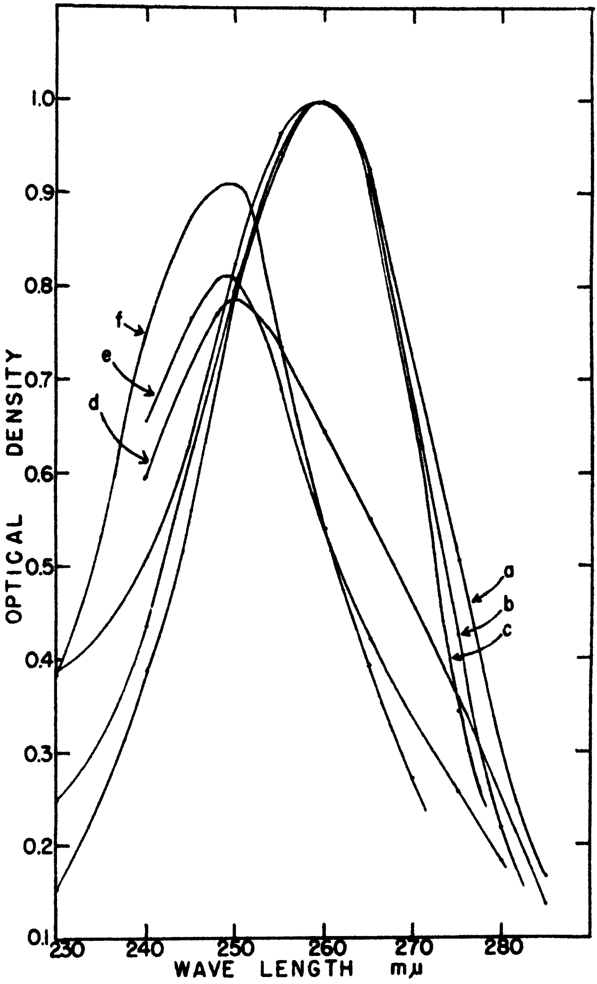

Fig. 2. Absorption spectra of sea urchin (*Lytechinus pictus*) adenosinetriphosphate, *a*, rabbit adenosinetriphosphate, *b*, adenosine, *c*, and the same substances after enzymatic dephosphorylation and deamination, *d*, *e*, and *f*. The adenosine and ATP values have been adjusted to a maximum density of 1.00. The spectra were obtained in 1.15 M phosphate buffer at pH 7.0, with a Beckman spectrophotometer.

of the barium insoluble phosphate from eggs of *Arbacia*, and that actomyosin fibers contract in the presence of this phosphate fraction, just as they do in the presence of adenosinetriphosphate.

It would be of some importance to find a specific tie-in between the energy-rich phosphate of adenosinetriphosphate and developmental processes, either mechanical or chemosynthetic. It is entirely possible, for example, that activation of the egg at fertilization includes an awakening of processes dependent on adenosinetriphosphate, or perhaps mitotic activity is dependent on energy supplied by phosphorylative mechanisms.

Attempts have been made by Runnström (1933) and Lindberg (1943) to find changes in the phosphate balance with fertilization in echinoid eggs which might reflect a change in activity of phosphorylative mechanisms. They report that no appreciable change in any fraction occurred. Barth and Jaeger (1947) have pointed out, however, that analysis for the concentration of a phosphate compound at various times during normal unmodified development might not reveal even large changes in the rate of turnover of the substance because of the likelihood that an increase in its utilization might be balanced by a corresponding increase in its formation, leaving the concentration at any time constant. By interrupting the events leading to adenosinetriphosphate synthesis in *Rana pipiens* gastrulae by imposing, among other things, anaerobic conditions on the embryos, they have been able to show that the adenosinetriphosphate of the eggs falls from 24.6 micrograms/100 embryos to 16 micrograms/100 embryos, concomitant with a partial inhibition of development. The demands of morphogenesis and maintenance are too much for the anaerobic adenosinetriphosphate synthesizing mechanisms. Developmental energy needs during the cleavage stages, however, are less demanding, and the anaerobic mechanisms are able to maintain the adenosinetriphosphate store nearly intact. These important experiments apparently represent the first demonstration that the energy-rich phosphate bond is closely linked to a developmental process.

Comparable experiments by the author designed to show an interdependence of fertilization and adenosinetriphosphate turnover in the eggs of *S. purpuratus* have so far been inconclusive, and at present the only reported interchange of phosphorus among the acid-soluble components of sea urchin embryos is that found by Zielinski (1939) in *Paracentrotus lividus* embryos poisoned with 0.025 M iodoacetate. In these, a small decrease in inorganic phosphate occurring progressively during the first two cleavages is accompanied by a corresponding increase in a phosphate ester that is probably propanediol phosphate. There was no alteration of adenosinetriphosphate concentration.

It will be remarkable indeed if no more significant interchange of phosphorus occurs in the fertilized echinoderm egg than is indicated by present knowledge in view of the great increase in respiratory activity accompanying activation in the echinoids, the close relation of respiration and mitosis established by Zeuthen, and the increased permeability of the fertilized egg to phosphorus shown by the radioactive isotope studies. Indeed, the mere presence of sizable amounts of adenosinetriphosphate and of an adenosinetriphosphatase to metabolize it (Connors and Scheer, 1947), leads one to expect a role for this ester in these important metabolic changes, though in view of the unusual array of acid-soluble phosphates present, it seems reasonable that the biochemical machinery to produce and utilize the energy-rich bonds may vary considerably from the familiar ones of muscle and yeast.

Attempts to illuminate this biochemical machinery in the echinoid egg have led to the view that the well-known Meyerhof-Embden scheme of muscle plays at most only a minor role. Runnström (1933, 1935) years ago found that neither sodium fluoride nor monoiodoacetate inhibited production of the acid of fertilization in sea urchin eggs, and Lindberg (1943) has established that glycogen and glucose breakdown in fertilized eggs or egg homogenates are not blocked by these inhibitors. Since the usual glycolytic mechanisms are inhibited by them, an alternative pathway of oxidation is indicated. This pathway apparently requires phosphorylative mechanisms, however, as Rothschild (1939) has found that phlorizin strongly inhibits the formation of acid of fertilization and, therefore, probably carbohydrate degradation (Lindberg, 1943).

An alternative mechanism to the Meyerhof-Embden scheme is that involving oxidation and decarboxylation of the hexose monophosphate molecule, carbon by carbon, a mechanism suggested by Warburg and Christian (1936) and Lipmann (1936) and developed further by Dickens (1938). Lindberg (1946) has proposed the working hypothesis that this pathway is the predominate one in the sea urchin egg. The suggestion is that hexose monophosphate is oxidized to phosphogluconic acid and this, in turn, to phosphoketogluconic acid. Proponediol phosphate is thought to act in this oxidation. The keto acid is then decarboxylated to form a pentose phosphate. Further oxidation presumably consists of a repetition of these steps.

The hypothesis is based chiefly on the following facts. He has tested the ability of egg homogenates to reduce methylene blue anaerobically in the presence of a number of possible substrates and finds that hexose phosphates, phosphogluconic acid, and propanediol phosphate are the most effective. The corresponding non-phosphorylated substances are ineffective. Further, pentoses increase in amount in the homogenates when propanediol phosphate is added and a small decrease in reducing sugar is found, a fact which could be explained by the oxidative decarboxylation of hexose phosphate. A further involvement of propanediol phosphate in respiratory metabolism is given by Lindberg's observation that the ester can stimulate respiration of the egg brei by as much as 100 per cent. A complete test of this working hypothesis will be of interest.

In addition to a possible role in energy metabolism in the sea urchin egg, propanediol phosphate and phosphogluconic acid have been found to have a morphogenetic effect in these eggs. Hörstadius and Gustafson (1947) have found both of these esters capable of animalizing isolated animal halves of early embryos of *Psammechinus miliaris* when applied in concentration of about 0.5 mg. per ml. Lactate and pyruvate have identical effects. The effect is expressed as an overdevelopment of the long cilia of the blastula and a suppression of development of the stomodaeum and ciliated band of the fully differentiated halves. Results have not been presented for the effect of the substances on vegetal halves. If Lindberg's hypothesis is correct, these results are in agreement with the suggestion of Lindahl (1936) that animalization is under the control of a gradient of carbohydrate metabolism highest at the animal pole.

LITERATURE CITED

Abelson, P. H
1948 *Biol. Bull.*, 95: 262.
Barth, L. G., and L. Jaeger
1947. *Physiol. Zool.*, 20: 133–146.
Brachet, Jean
1945. "Embryologie Chimique." Masson et Cie. Paris.
Brooks. S. C.
1943. *Biol. Bull.*, 84: 213–225.
Brooks, S. C., and E. L. Chambers
1948 *Biol. Bull.*, 95: 262–263.
Caspersson, T.
1947. *Symp. Soc. Exp. Biol.*, 1: 127–151.
Chambers, E. L , and W. E. White
1948. Personal communication.
Chambers, E. L., and A. H. Whiteley
1949. Unpublished experiments.
Chambers, E. L., A. Whiteley, R. Chambers and S. C. Brooks
1948. *Biol. Bull.*, 95: 263.
Connors, W. M., and B. T. Scheer
1947. *Jour. Cell. Comp. Physiol.*, 30: 271–284.

Crane, R. K.
1947. *Biol. Bull.,* 93: 192–193.
Dickens, F.
1938. *Biochem. Jour.,* 32: 1626–1644.
Goldinger, J. M., and E. S. G. Barron
1946. *Jour. Gen. Physiol.,* 30: 73–82.
Hall, T. S., and F. Moog
1948. *Jour. Exp. Zool.,* 109: 339–354.
Harvey, E. B.
1941. *Biol. Bull.,* 80: 354–362.
Harvey, E. N.
1932. *Biol. Bull.,* 62: 141–154.
Hörstadius, S., and T. Gustafson
1947. *Zool. Bidv. Uppsala,* 25: 571–581.
Jandorf, B. J., and M. E. Krahl
1942. *Jour. Gen. Physiol.,* 25: 749–754.
Kalckar, H. M.
1944. *Science,* 99: 131–132.
1947. *Jour. Biol. Chem.,* 167: 445–459.
Krahl, M. E., B. J. Jandorf and G. H. A. Clowes
1942. *Jour. Gen. Physiol.,* 25: 733–747.
Krahl. M. E., A. K. Keltch and G. H. A. Clowes
1940. *Proc. Soc. Exp. Biol. Med.,* 45: 719–721.
Lindahl, P. E.
1936. *Acta Zool.,* 17: 179.
Lindberg, Olov
1943. *Ark. Kemi, Mineral., Geol.,* 16: A, No. 15, 1–20.
1946. *Ark. Kemi, Mineral., Geol.,* 23: A, No. 2, 1–45.
Lipmann, F.
1936. *Nature,* 138: 588–589.
Mitchell, H. K., and Wm. D. McElroy
1946a. *Arch. Biochem.,* 10: 343–349.
1946b. *Arch. Biochem.,* 10: 351–358.
Needham, J.
1942. "Biochemistry and Morphogenesis." Cambridge University Press.
Needham, J., and D. M. Needham
1930. *Jour. Exp. Biol.,* 7: 317–348.
Rothschild, Lord
1939. *Jour. Exp. Biol.,* 16: 49-55.
Runnstrom, J.
1933. *Biochem. Zeit.,* 258: 257–279.
1935. *Biol. Bull.,* 69: 345–350.
Schmidt, G., L. Hecht and S. J. Thannhauser
1948. *Jour. Gen. Physiol.,* 31: 203–207.
Tyler, Albert
1942. *Quart. Rev. Biol.,* 17: 197–212, 337–353.

Villee, C. A., M. Lowens, M. Gordon, E. Leonard and A. Rich
1948. *Biol. Bull.,* 95: 279.
Warburg, O., and W. Christian
1936. *Biochem. Zeit.,* 287: 440.
Zeuthen, E.
1949. This conference.
Zielinski, M. A.
1939. *Acta Biol. Exp.,* 13: 35–48.

DISCUSSION

CHAIRMAN ENGLE: Thank you, Dr. Whiteley. The occurrence of phosphorus uptake in metabolizing tissues was shown to me for the first time in developing eggs, and there is great interest in this technique. I will now turn this paper over to the conference for discussion.

DR. ZEUTHEN: You mentioned that in Barth's work there was a difference between the first period of development and the later divisions in the speed of restoration of ATP. At what time does the difference show up?

DR. WHITELEY: In the amphibian study the early gastrulae were made anaerobic for twenty-four hours, and during this time they started losing ATP and continued to lose it. During the cleavage stages, stage 8, as I remember, the decrease in ATP was considerably less. Apparently the eggs could handle their smaller morphogenetic problems at that time.

DR. ZEUTHEN: Nobody has tried to investigate the cleavage period to see if there are differences within the cleavage period?

DR. WHITELEY: I know of no such study. Barth and Jaeger studied an extended period involving several cleavages in the frog egg.

DR. HAAS: As I recall, early experiments of Warburg demonstrate that the respiration of the egg, after fertilization, increases sharply. The respiration can be inhibited by cyanide and it seems to resemble also in every other respect the normal pattern of respiring cells. Wouldn't it be important then to consider in the developing egg the role of the phosphorylated carbohydrates which are known to participate as intermediates in respiratory and fermentative processes?

DR. WHITELEY: It certainly would be important to look intensively for all the usual phosphorylated intermediates. None of them, other than those discussed, has so far been found in the eggs. One would certainly expect to find more than just these few, but any other phosphate compounds that may be present must be in low concentration, judging from the data of the table presented. Very little acid soluble phosphate is left over after one takes into account the three components discussed.

DR. HAAS: With enzymatic methods it may be possible to detect phosphorylated intermediates which are too unstable or which might be present in too low a concentration to be observed by conventional method. An enzymatic method, for example, is available to determine accurately as little as 10 micrograms of glucose-6-phosphate.

DR. WHITELEY: Enzymatic techniques are certainly the ones to turn to. Glucose-6-phosphate may well be present, but the procedure of analyzing for its barium salt precipitated out of alcoholic solution doesn't give evidence of its presence in this material. Other compounds undoubtedly are also present and their measurement merely waits on the use of sufficiently sensitive techniques. Your enzymatic method for glucose phosphate sounds like a very good one to apply.

DR. SPIKES: Of course it should be pointed out that it is not a general phenomenon that respiration increases after fertilization. In many animals it drops as quickly as it increases in the sea urchin.

CHAIRMAN ENGLE: Are there any other pertinent comments?

DR. CHAMBERS: One of the most striking things about the uptake curve is the extraordinary difference between the unfertilized and fertilized eggs. It is 160 times as rapid in the fertilized eggs sixty minutes after fertilization than in the unfertilized egg. There was one further question I wanted to ask Dr. Whiteley. We got two milligrams of adenosinetriphosphate per gram of Arbacia egg. I wonder how that would compare with your data. I can't figure it out in my head.

DR. WHITELEY: The agreement between your data and mine is very close, and further, both are in good agreement with the values found for rabbit muscle. Specifically, I find 2.2 mg. of the barium salt of ATP per gram wet weight, or 1.3 mg. of the free acid, in *Strongylocentrotus purpuratus*. The corresponding figures for *Lytechinus pictus* are 3.6 and 2.1. Yields from rabbit muscle run about 3.5 mg./gram wet weight.

CHAIRMAN ENGLE: Going back to this question, I would like to ask Dr. Zeuthen a question. I don't want to presume on your presentation later. Are you going to discuss the role of phosphorus here in relation to oxygen consumption?

DR. ZEUTHEN: No, I am not. I am going to discuss the segmentation period, meaning the period from the first up to the ninth division or so, and the question asked Dr. Whiteley was whether people have bothered with analyzing those periods. Most people are interested in taking samples of what they are interested in over much too long intervals to give any kind of information which can be made, at the present time, to fit into the picture of fertilization.

CHAIRMAN ENGLE: I take it, then, that none of you are committed to a definite statement as to whether the oxygen consumption is definitely correlated with this increase in phosphorus uptake.

DR. WHITELEY: I imagine it would be safe to say there is some correlation somewhere, but how it is related is not known. Not all of the intermediates of the Krebs cycle are known to be present in the echinoderm egg. The metabolic system here is apparently quite different from that of many other tissues.

Dr. Tyler: It certainly would be worth-while to make comparative studies of species, such as Chaetopterus and Cumingia, in which there is a drop in oxygen uptake upon fertilization.

Chairman Engle: Is there any further comment?

Dr. Whiteley: I would like to make a comment in connection with Dr. Moore's talk. He mentioned there were two precursor substances for the fertilization membrane which apparently had to be gotten rid of before the egg can divide. I have tried to block cleavage of echinoderm eggs with anaerobic conditions, but I have been uniformly unsuccessful in getting anaerobic blocks. However, it is true that anaerobic conditions applied at the time of fertilization will block it, and I wonder if the difference in effect of anaerobiosis might not be related to the precursor substances. The failure of the membrane to lift anaerobically would cause retention of the substance by the egg, and therefore prevent cleavage, whereas later the substances would have left the egg, thus possibly making it less sensitive to anaerobiosis.

Dr. Moore: It is a most interesting suggestion from entirely different angles. That seems very reasonable.

Dr. Scott: I want to ask you what you mean by the statement that the development is not blocked by anaerobic conditions. I agree that it is not entirely blocked by anaerobic conditions, although I know that the common belief is that it is. I have been really working on the cytological aspects of anaerobiosis. I think it is going to take a while to develop a good anaerobic technique. Still we are down to a very low oxygen level. I find mitosis creeping along in the best anaerobic conditions. I would like to know what your criterion was.

Dr. Whiteley: Mainly the delay in cleavage time. I have had uniformly bad luck in causing the first cleavage to be delayed by anaerobic conditions. Maybe the anaerobic conditions weren't thorough enough, though luminescent bacteria blacked out within a minute under the conditions used. The eggs go ahead and are not delayed.

Dr. Scott: I must say I get delay. I have passed the nitrogen over hot copper.

Dr. Whiteley: I have used that.

Chairman Engle: Is there any other comment? If not, we will move on to the next paper by Dr. Edward L. Chambers, of the University of California, Berkeley, on "Ionic Exchanges and Fertilization in Echinoderm Eggs."

(Conference concluded in the issue of November–December)

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CONFERENCE ON PROBLEMS OF GENERAL AND CELLULAR PHYSIOLOGY RELATING TO FERTILIZATION. III.[1]

ION EXCHANGES AND FERTILIZATION IN ECHINODERM EGGS[2]

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THIS lecture summarizes work initiated by the second author and continued over several years by the two authors particularly on *Arbacia punctulata* (Chambers and Chambers, 1938, 1939; Chambers, 1946). Further investigation on the subject was done by the first author in conjunction with the late Dr. S. C. Brooks, using the eggs of two species of *Strongylocentrotus* with radioactive ions as tracers (Chambers *et al.*, 1948; Chambers, 1949).

The experiments constitute an analysis of the changes in susceptibility of the developing sea urchin egg to isotonic potassium chloride. These data are considered in the light of studies on the rate of exchange of potassium and sodium in the sea urchin egg.

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[1] Sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949. Conference Chairman, Earl T. Engle, Columbia University, New York. Program Secretary, Albert Tyler, California Institute of Technology. Representative of the N.R.C., Deborah C. Leary, New Haven Hospital, New Haven.

[2] Aided by a grant from the National Cancer Institute, U. S. Public Health Service.

1. EFFECT OF KCl ON THE UNFERTILIZED AND FERTILIZED EGGS OF *Arbacia punctulata*

Eggs were transferred to isotonic KCl, unbuffered, at pH 6.0, 18° C., through several washes by centrifugation. Unfertilized eggs were immersed in the KCl, and returned to sea water at intervals of time and tested for their viability by inseminating them and securing the percentage cleavages. After 8 hours' immersion in KCl, 98 per cent. of the unfertilized eggs were fertilizable and then cleaved. Longer sojourns resulted in increasingly lower percentages of cleavage.

Transference of the fertilized eggs to KCl caused at certain times a complete cessation of further development and not at others. In accordance with the reaction observed on immersion three definite periods were found—an early and a late susceptible period characterized by inhibition of development, and an intermediate resistant period during which development continued. A remarkable feature is that eggs transferred to KCl during the resistant period continue on to cleavage at least beyond the 32-celled stage. This must mean that by virtue of their having been immersed in KCl early, they have acquired the ability to pass through the late susceptible period.

Another feature of interest is the speed of the KCl effect during the susceptible periods. Thus, in the early susceptible period, which is of short duration, further development immediately stopped. Also, during the late susceptible period when the eggs are placed in the KCl a minute or two before cleavage only a small percentage cleave and these cleavages are highly irregular.

Fig. 1 shows the per cent. of eggs which underwent cleavage after they had been immersed in KCl at different intervals. The abscissae represent times after insemination, the ordinatés, the percentage cleavage.

(*a*) 0.5′ to 1.5′ After Insemination: The time denotes the moment when the eggs first came into contact with the KCl solution. These eggs were examined at various periods of time, and it was found that the immersion in KCl had immediately arrested further development.

In some, the head of a spermatozoon could be seen at the cortex of the egg, with its tail extended straight and motionless outside. Various stages of the lifting of the fertilization membrane were also observed. In some of them the elevation was complete. In regard to the position of the pigment granules the eggs exhibited the characteristic unfertilized state in which the granules lie scattered throughout the interior of the egg. Some of these eggs were continuously observed for half an hour with no sign of motion, either of further entry of the spermatozoon, lifting of the membrane, migration of pigment granules or of the egg pronucleus. The eggs were returned at various intervals of time into sea water, and, up to the period of one-half hour, the fertilization process was resumed and the eggs underwent normal first cleavage. The only observable difference between these and eggs which had been continuously in sea water was a delay in time corresponding with the period of sojourn in the KCl solution and the absence in many cases of a fertilization membrane. The ability of these eggs to develop after return to sea water was found to be as follows: After half an hour's sojourn the cleavage was normal and occurred about half an hour after the controls. The hyaline layer (hyaline plasma layer) was sufficient to hold the blastomeres together. After one and one-half hours'

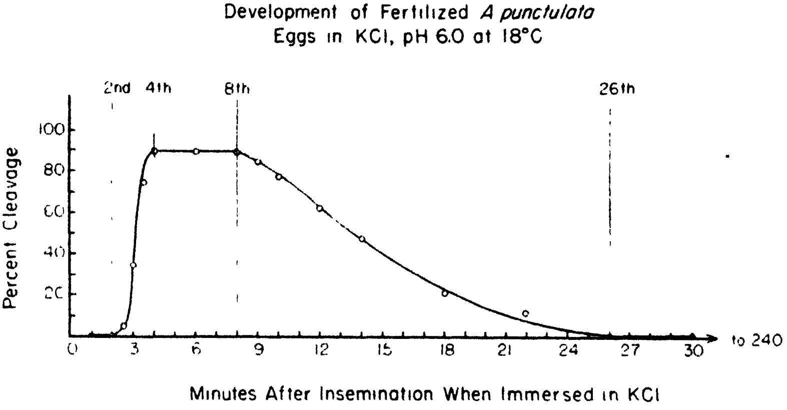

FIG. 1.

sojourn the viability of the eggs was definitely impaired, and cleavage was abnormal with a slight hyaline layer or in some cases it was absent. After two hours' sojourn the eggs in sea water maintained the unfertilized appearance of their interior and underwent no further development. They continued healthy in appearance as long as do normal unfertilized eggs in sea water. These eggs, however, could not be refertilized.

(*b*) 2.0′ to 3.5′ After Insemination: Eggs immersed in KCl 2.0′–3.5′ after insemination lost their fertilization membranes, although many continued development and cleaved to a variable degree. Those immersed at 2.0′–2.5′ cleaved in small percentages, while those immersed at 3.0′–3.5′ cleaved in large percentages, but mostly not beyond the 2-celled stage. The eggs which did not cleave either remained in an apparently unfertilized state or developed only to the streak or amphiaster stage. A small per cent. may develop as far as the 32- or 64-celled stage, the cells being non-coherent.

(*c*) 2.0′–4.0′ to 8′–13′ After Insemination (Resistant Period): All these develop in high percentages regularly (95 to 98 per cent.) through the 64–128-celled stages. The pigment granules remained intact in their usual position at the periphery of the egg and did not disappear throughout subsequent cleavages. The formation of the typical sperm aster and the subsequent development of the amphiaster occurred as in the normal egg.

Those immersed at 4′ lost the fertilization membrane and showed no sign of a hyaline layer. The blastomeres either fell apart or remained connected each by a long slender intercellular bridge. They appeared as colonies of loosely aggregated cells. Those immersed from 4.5′–8′ maintained their fertilization membrane and the developing blastomeres remained together but not coherent, and there was no sign of a hyaline layer. These blastomeres lay at random. Some of these at the 64–128-celled stage were transferred to sea water. The spaces among them exhibited a fine granular precipitate, presumably of precipitated hyaline layer material, which had been retained in the dispersed state within the fertilization membrane. Many of the aggregates developed into typical blastulas which continued their development into swimming embryos. On being slightly torn with micro-needles, the embryos readily fell apart, indicating that whatever hyaline material had formed in sea water was very weak and fragile. Eggs immersed in KCl during this period are designated KCl-*resistant* eggs, since they are able to develop in KCl through all further stages, at least up to the 64–128-celled stage. On return of the KCl eggs to sea water at any time up to six hours after immersion, the eggs exhibited complete recovery and continued normal development. The sojourn in KCl had not caused any permanent damage.

(*d*) 8′–13′ to 15′–26′ After Insemination: These eggs underwent development to a variable degree. Those immersed earliest cleave in large percentages, whereas those immersed later cleave in smaller percentages. The eggs remaining single may or may not develop through to later stages. These eggs which cleave may develop only as far as the two-celled stage.

(*e*) 15′–26′ and Later After Insemination (Susceptible Period): Immediately upon immersion in KCl further development ceased. This was found to be true for the eggs immersed at frequent intervals of time up to 240′ after insemination and at the 32- to 64-cell stage. No rhythm of resistance and susceptibility could be detected during successive cleavage stages. The immediacy of the cessation was demonstrated by the fact that the eggs just before cleavage exhibiting karyokinetic lengthening were stopped at this stage by the immersion.

The astral radiations of eggs in the monaster or amphiaster stage disappear within 10′ after immersion. Most of the pigment vacuoles, all of which lay in the periphery, disappeared, leaving the eggs relatively colorless. The hyaline lake enclosing the amphi-nucleus of the monaster stage persisted until cytolysis eventually occurred 2 to 3 hours later. Complete recovery was possible if the eggs were returned to sea water within 30′ and the delay in cleavage time approximated the length of sojourn of the eggs in KCl.

Of these eggs which were immersed at the height of the sperm aster and the amphiaster of the first cleavage, the cleavage time was definitely prolonged over that of the eggs which had been placed in KCl during the interkinetic stages.

The recoverability of eggs immersed in KCl for a longer period than 30′ was characterized by the exhibition of a periodic fluctuation. The relation of this fluctuation to the particular stage when the eggs were transferred to KCl is indicated as follows: When the eggs were placed in KCl at the height of sperm aster development, the recoverability in sea water was very low, as indicated by an extremely small percentage which cleaved. Among these, where cleavage did occur, the time was greatly prolonged beyond the length of the previous sojourn in KCl. When the eggs were placed in KCl during the streak stage, the recoverability was high. In these cases the delay in cleavage time more nearly approached the length of previous sojourn in KCl. Where the eggs were immersed in KCl solution at the height of development of the amphiaster, just prior to cleavage, the recoverability again fell, to rise during the interkinetic stage between first and second cleavage. In all these, the delay in cleavage time was over that which would be expected as a reflection of the length of sojourn in KCl.

The difference between the early period from 0′–2.0′ after insemination and the susceptible period should be emphasized. Eggs immersed in the KCl solution within the first 2.0′ maintain a healthy appearance long after eggs immersed during the susceptible phase have undergone complete cytolysis. The block to development during the early period is probably due to an effect of the KCl on the spermatozoon, whereas during the susceptible period the block is exerted directly upon the egg.

2. Effect of K Salts Other than the Chloride on the Fertilized Eggs of *A. punctulata*

The salts used were sulfate, formate, acetate, citrate, oxalate and tartrate. These were all adjusted to pH 7.0 without buffer. The salt solutions were isotonic with sea water, and no change in diameter of spherical eggs occurred after immersion.

The sulfate, citrate, oxalate and tartrate all revealed resistant periods similar to the chloride, and development during the resistant phase was superior to that in KCl. The formate and acetate were highly toxic and inhibited development at all periods.

3. EFFECT OF KCl ON THE EGGS OF *Strongylocentrotus purpuratus* AND *S. franciscanus*

Essentially the same phenomena were observed in these eggs (Fig. 2). However, an important difference between these eggs and those of *A. punctulata* is that rhythmic periods of resistance are observed prior to both first and second cleavages.

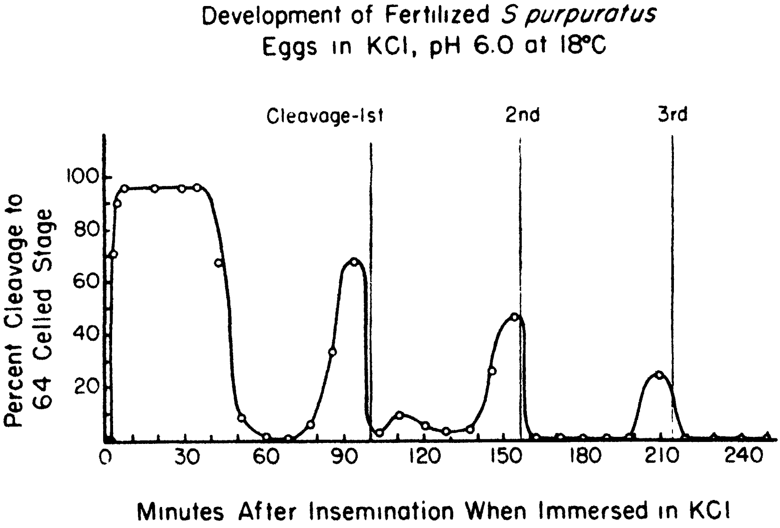

FIG. 2.

4. SALTS WHICH COUNTERACT AND SALTS WHICH SUSTAIN THE SPECIFIC KCl RESISTANCE OF ARBACIA EGGS

(*a*) Sea Water: Eggs inseminated in sea water were transferred within five minutes into a solution of KCl, pH 6.0 at 18° C. A quantity of the eggs was removed 25′ later

(30′ after insemination) exposed to sea water for 1′ and returned to KCl. These eggs failed to develop, indicating that a brief exposure to sea water of eggs which had been in KCl, will, in so far as susceptibility is concerned, make them react as if they had been continuously in sea water. It is to be noted that the time of 30′ when they were transferred to KCl is the time when the eggs would be expected to be susceptible to KCl.

(*b*) Individual Salts of Sea Water: The next step was to ascertain which of the salts present in sea water are responsible for this effect. In order to arrange this experiment, it was necessary first to ascertain the effect of continuous immersion in each of the salts to be tested.

Accordingly there were prepared isotonic solutions, at pH 6.0 and pH 8.0, of NaCl, Na_2SO_4, $MgCl_2$ and $CaCl_2$. Eggs inseminated in sea water were transferred 25′ later into each of the above salt solutions. Eggs in the NaCl, Na_2SO_4 and $MgCl_2$ at pH 6.0 and 8.0 failed to undergo cleavage. Eggs exposed to $CaCl_2$ at pH 6.0 also failed to cleave, but those in $CaCl_2$ at pH 8.0 underwent cleavage at least up to the 4-celled stage.

The retransfer experiment with KCl was now repeated. Eggs were placed in KCl 5′ after insemination, left there for 25′ and then exposed for 2′ and 10′ to each of the sodium and magnesium salt solutions after which they were brought back to the KCl solution.

(1) NaCl, Na_2SO_4 and $MgCl_2$: The eggs exposed to the NaCl, Na_2SO_4 and $MgCl_2$ at pH 6.0 and 8.0 continued to develop in the KCl solution with the exception that the longer sojourn of 10′ resulted in the non-development of a certain percentage of the eggs. The probability that this is due to the toxicity of the longer sojourn was tested in the following experiment.

The length of the sojourn of the eggs in the above four salt solutions was varied and some of the eggs returned to sea water at the same time that the remainder were being placed back in the KCl solution. It was found that the percentage of eggs which now failed to develop in the KCl was the same as in the sea water. It was therefore concluded that whatever failure of development occurred in the KCl solution was due to the toxic effect of the sojourn in Na and Mg salt solutions. Tests were also made with various mixtures of NaCl and $MgCl_2$ at pH 6.0 and 8.0. No difference was found from the action of these salts used individually.

The fact that the eggs which were not affected by immersion in these salt solutions continued to develop on retransfer to KCl indicated that none of these salts could be responsible for the action of the sea water on the KCl resistance.

(2) $CaCl_2$: The remaining salt to be tested was $CaCl_2$. It was found that a transfer at 25 minutes of the KCl-resistant eggs to the $CaCl_2$ (either pH 6.0 or 8.0) for as short a time as 1′ produced the same effect as the sea water in stopping further development upon return of the eggs to KCl. The eggs left continuously in the pure $CaCl_2$ developed through the 8-cell stage.

Experiments were then made to ascertain the minimum concentration of $CaCl_2$ which would induce this specific effect. The calcium was diluted with isotonic NaCl solution. The specific action of the $CaCl_2$ was evident in dilutions down to 0.002 M $CaCl_2$ in .52 M NaCl. With further dilutions, *e.g.*, .001 and 0.005 M $CaCl_2$, the effect of the mixture increasingly approximated that of NaCl alone. The eggs may be placed at any time in a salt solution at pH 8.0 of .51 M NaCl and .01 M $CaCl_2$, in the same proportion as occurs in sea water, and normal cleavage follows. However, the development does not continue as well as in sea water in which the other essential ion is K and possibly Mg. The concentration of $CaCl_2$ may be progressively decreased down to .002 M, without significantly impairing development. Below this concentration the toxicity increases, until at .0005 M $CaCl_2$ the effect approximates that of pure isotonic NaCl.

Dilutions were also made of the $CaCl_2$ with .53 M KCl. In these mixtures the specific action of the Ca was lost only at concentrations below 0.08 M $CaCl_2$.

(*c*) Other Salts—RbCl, LiCl, $BaCl_2$ and $SrCl_2$: Analogous experiments were made using Rb, Li and Ba. These gave results similar to the results with Na and Mg. Experiments using Sr gave results similar to those of Ca, except that the Sr ion was less effective in destroying KCl resistivity.

From these experiments the conclusion may be drawn that the ability of Arbacia eggs to develop in pure KCl when immersed early is due to the lack of the calcium ion. The presence of calcium in sea water is responsible for the development of susceptibility to the isotonic KCl.

5. THE EXCHANGE RATE OF K IN THE EGGS OF *S. purpuratus* AND *A. punctulata* IMMERSED IN SEA WATER

We next became interested in finding what relation the observed periods of resistance and susceptibility to KCl might have to differences in ion exchange during different stages of development. The research was then brought to the laboratory of the late Dr. S. C. Brooks, and radioactive ions were used to measure ion exchange rates. Our objective was to find whether differences in ion exchange could be detected in the eggs at various stages of development.

A dilute (.2 per cent.) suspension of unfertilized eggs was prepared, and an equal quantity added to each of two beakers. Carrier free K^{42} was then added and, simultaneously, the eggs in one beaker were inseminated, while the eggs in the other beaker were left unfertilized. The uptake of K^{42} was then followed by taking frequent samples for radioactivity measurements. Correction was made in each sample for the amount of radioactivity present in the suspension fluid surrounding the eggs. Samples of eggs and suspension fluid were also taken for determinations of the K content. The results for K^{42} appear in Fig. 3. The ordinates represent counts per minute per ml. of eggs, and the abscissae represent time in minutes after addition of K^{42}. In the case of the fertilized eggs this is the same as time in minutes after insemination. The unbroken and interrupted lines represent, respectively, the uptake of K^{42} by the fertilized and unfertilized eggs. The dot-dash line shows the level of activity in the suspension fluid per ml. The crosses U, V and W, and X, Y and Z indicate the times when samples were taken for analyses of the total K content. The K content in the fertilized eggs remained constant from the time of insemination, at U, to 1000′, at Y. The K content of the unfertilized eggs remained constant through 500′ at V, but thereafter a fall was observed at W. The rate of uptake of K^{42} by the unfertilized eggs is very slow, while in the fertilized eggs the rate is very much more rapid. It is important to note that a marked increase in uptake of K^{42} by the fertilized eggs is observed long before first cleavage (see Fig. 3). However, the increased uptake of K^{42} does not reach a maximum until 40′–60′ after insemination. Fig. 3 is drawn on too large a scale to show this.

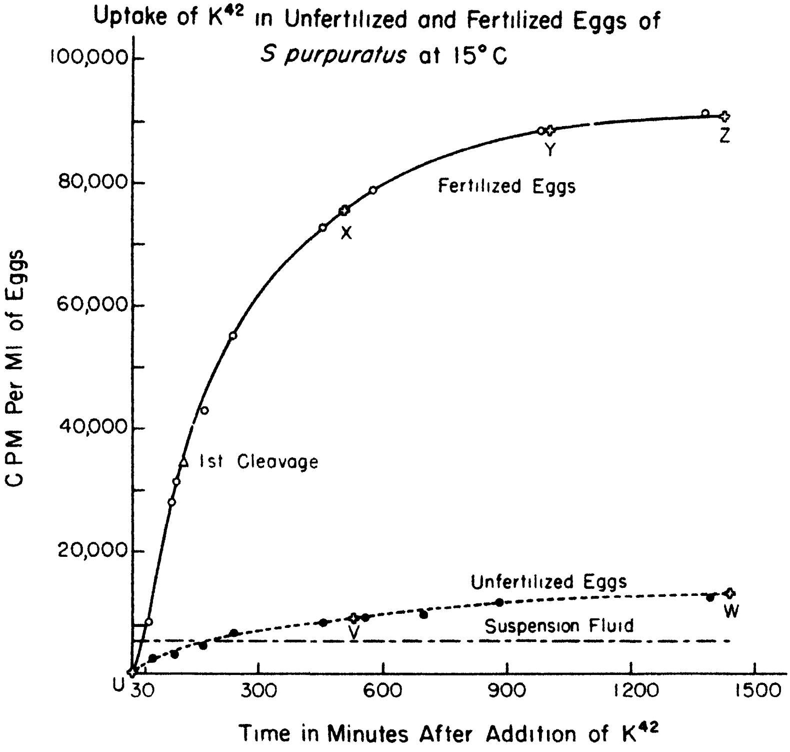

FIG. 3.

Similar experiments were performed using the eggs of *A. punctulata,* and the same results were obtained.

A study of the rate of exchange of K^{42} in eggs immersed in sea water reveals, therefore, that the exchange rate is very slow in the unfertilized egg. After fertilization, the exchange rate becomes very rapid. This increase does not become maximal until a definite period after fertilization.

6. THE EXCHANGE RATE OF K AND Na IN THE EGGS OF *A. punctulata* IMMERSED IN KCl

The next step was to determine the exchange rate of K and Na in pure KCl of the unfertilized eggs and of eggs in the resistant and susceptible phases of development. Radio-

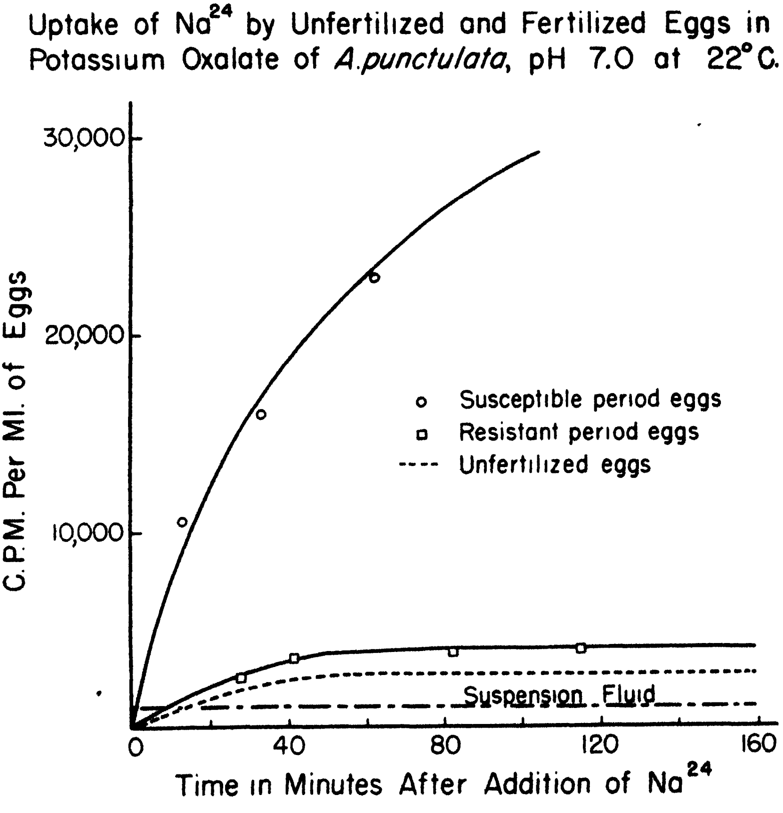

FIG. 4.

active K^{42} was added to a suspension of unfertilized eggs in sea water. After the eggs had taken up sufficient K^{42}, a portion of them was inseminated. The eggs containing the K^{42} were then placed in isotonic KCl and the rate of loss measured. It was found that the rate of loss of K^{42} in unfertilized eggs and in eggs immersed in KCl within 2.0′ after insemination was relatively slow, and somewhat more rapid in the resistant period eggs. In the susceptible period eggs, however, the loss was very rapid, all of the K^{42} having diffused out within 1′ to 2′ after immersion of the eggs in KCl.

The experiment was also performed using Na^{24}. Minute quantities of NaCl containing Na^{24} of high specific activity were added to suspensions of eggs in pure KCl. One suspension was of unfertilized eggs, the second of fertilized eggs in the resistant phase, and the third of fertilized eggs in the late susceptible phase. The rate of uptake of Na^{24} was measured in each of the three suspensions (see Fig. 4). The uptake of Na^{24} by the unfertilized eggs and the resistant, fertilized eggs was negligible, whereas a rapid uptake of Na^{24} was observed in the susceptible period eggs.

7. CONCLUSION

The experiments with radioactive ions indicate that when eggs are transferred from sea water to KCl, the relative differences in rates of ion exchange observed in sea water are maintained. The extraordinary feature is that fertilized eggs immersed in pure KCl during the resistant period have a low rate of ion exchange in spite of continued development in the solution.

LITERATURE CITED

Chambers, Edward L., and Robert Chambers
1938. *Biol. Bull.*, 75: 356.
1939. *Anat. Record*, 75: 44.
Chambers, Edward L.
1946. *Anat. Record*, 94: 372.
1949. *Biol. Bull.*, 97: 251.
Chambers, Edward L., W. White, N. Jeung and S. C. Brooks
1948. *Biol. Bull.*, 95: 252.

DISCUSSION

CHAIRMAN ENGLE: It must be wonderful where you can work with organisms and a bucketful at very little cost. I am amazed at the uniform precision with which you can make observations. Dr. Chambers's paper is open for discussion.

DR. ZEUTHEN: Have you any opinion whether the potassium ion is in some fixed combination in the cytoplasm or not? In the former case the exchange would measure the rate at which ions are released from such fixed combinations. In the latter case the exchange rate must depend on the permeability of the plasma membrane.

DR. CHAMBERS: That is a highly debatable point. Our impression is that ion exchange rates are determined by the forces which attract or hold the ions to the proteins and other structures of the cell. It is more a question of the ability of the ions to get away from these forces than the ease with which they are able to cross the membrane.

DR. MOORE: There is a point which I think might be looked out for, and that is the potassium development which Frank R. Lillie showed years ago. He obtained gastrulae without cell division in sea water containing an excess of potassium. I thought possibly you might have observed the internal changes without cell division.

DR. CHAMBERS: In our experiments the susceptible and resistant phases were concerned with eggs exposed to KCl completely free of sea water. In the susceptible period the cessation of development was of the cell as a whole including its nucleus. Dr. Lillie's findings seem to have no relation to ours.

DR. MOORE: Do you relate your findings to the other rhythmical changes that have been noted?

DR. CHAMBERS: The significant feature of this work is that by virtue of immersing the eggs in KCl at an early stage of development, they now have the ability to pass through later highly susceptible periods. This phenomenon bears no apparent relationship to the earlier work on rhythmicity. Those experiments mentioned in the body of the paper in which the development of Arbacia eggs in sea water was observed after a period of immersion in KCl at various stages of development, however, are comparable. The Arbacia egg is susceptible, upon transfer from sea water to KCl, during periods of marked astral development (monaster, amphiaster). It is resistant for a short period immediately after insemination and during the resting phases of development. In the experiments with KCl, except for two short transitional periods, development in the KCl either continued unimpeded or it was immediately stopped. Furthermore, at no time did the KCl appreciably alter the volume of the eggs. These results are similar to those of Lyon (1902) who placed eggs in sea water to which KCN was added, and in sea water at 0° C. at various intervals of time after insemination. The experiments with KCl are directly comparable to Lyon's experiments, since in both cases the volume of the eggs was unaltered, and development was immediately stopped by the immersion.

The increase in susceptibility of eggs immediately prior to and during cleavage to hypotonic sea water (R. S. Lillie, Just), hypertonic sea water (Moore), and higher alcohols (Baldwin), and ether (Spaulding) has been demonstrated. In experiments of this type, which involve a considerable alteration in cell volume, such physical factors as the consistency of the protoplasm and its various components, and the extraneous coats undoubtedly play an important role in determining the resistance of the egg.

DR. TYLER: When the eggs develop in pure KCl in the early stages, is the development relatively quite normal? And is the hyaline plasma layer formed?

DR. CHAMBERS: The development of the eggs in the KCl is quite normal for at least the first three cleavages, with the one exception that the KCl eggs apparently retain all their pigment vacuoles. In normal development in sea water, there is a progressive diminution of the pigment vacuoles in the cortex. The hyaline layer is not visible in eggs developing in KCl. If the fertilization membrane has been removed before immersing the eggs in KCl, the complete nakedness of the surface of the egg can be demonstrated by the oil coalescency method using a micropipette. However, if eggs which retain their fertilization membrane are allowed to develop in KCl, and are then replaced in sea water, a fine, diffuse granular precipitate forms between the surface of the egg and the fertilization membrane. This precipitate undoubtedly is due to the interaction of calcium with the hyaline plasma layer material, which had been formed in the KCl, but had remained in a soluble state within the confines of the fertilization membrane.

DR. TYLER: Is fertilization possible in KCl? I think you have tried that.

DR. CHAMBERS: Spermatozoa are immediately immobilized in KCl at pH 6.0, and fertilization of the eggs is impossible. I am interested in what you think about the initial susceptible phase to KCl. Do you think this represents the inhibitory action of the KCl directly on the sperm?

DR. TYLER: It wouldn't take more than 15 to 20 seconds for the sperm to enter.

DR. CHAMBERS: Yet the initial susceptible phase lasts two minutes. During this phase the sperm remains at the surface of the egg.

DR. TYLER: Several people, including myself, now think that the establishment of the block to polyspermy may be a rather slow affair, and that various sperms start into the egg, but after a short time all but one of them are repelled. There is some recent evidence concerning this which Dr. Rothschild has accumulated in studies of the cortical changes upon fertilization. In contrast to earlier workers he finds that these changes are relatively slow.

METABOLISM OF SEA URCHIN SPERM[1,2]

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THE physiology of fertilization, at least for convenience, may be investigated from two aspects with regard to the participation of the spermatozoon. First, the process by which the sperm reaches the egg must be studied. In most species of animals the sperm is motile; thus it becomes necessary to determine how the energy for motility is obtained. This involves a study of the metabolism of the sperm cell. The second aspect concerns the mechanism by which the sperm attaches to and penetrates into the egg after reaching its surface. It is probable that sperm motility is not necessary for this second process. The metabolism of the sperm of certain vertebrates, especially mammals, has been extensively studied, but little is known of the interactions between the sperm and eggs of vertebrates. Conversely, remarkably little work has been published on the sperm metabolism of invertebrates, while extensive work has been done on the interactions between the sperm and eggs of certain invertebrates.

Materials used in this work were sperm of the Pacific coast sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus pictus*. The results obtained with the two species were essentially the same, although certain quantitative differences were observed. The Warburg technique was used for measuring oxygen consumption and carbon dioxide production.

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[1] This work was carried out at the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena.

[2] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena. January 22, 1949.

Mann (1946) showed that the seminal fluid of most vertebrates contained fructose and that this was the normal substrate of the sperm. Lardy and Phillips (1941) showed that bull sperm deprived of external substrate were capable of oxidatively metabolizing endogenous phospholipid. It was thus of interest to determine the normal substrate of sea urchin sperm, since sea urchin sperm leads a somewhat different life from mammalian sperm. It is shed free into the sea water and is thus highly diluted. As a result, even if there were a substrate present in the so-called seminal fluid it would not be of much use to the sperm. The sea urchin sperm therefore has to carry its own total supply of substrate.

The respiratory quotient of sea urchin sperm is approximately one. This indicates that they are metabolizing carbohydrate. A better way to determine the substrate utilized is to perform analyses on the sperm for a number of possible substrates before and after aging the sperm under controlled conditions. The results of a typical experiment of this kind are shown in Table 1. As may be seen, the only constituent showing a marked change with aging is the reducing value of the glycogen-like fraction. This agrees with the value of the respiratory quotient. It would seem therefore that the normal substrate of the sperm is a glycogen-like substance.

TABLE 1

CHEMICAL CHANGES IN STRONGYLOCENTROTUS SPERM DURING 12 HOURS OF AGING AT 19 DEGREES CENTIGRADE

(Expressed in mg. per gm. dry wt. of sperm)

Constituent	Before aging	After aging
Reducing value, total	80.6	9.8
Reducing value, TCA sol.	1.4	0.7
Reducing value, glycogen-like material	29.0	8.5
Total lipid	104	107
Neutral lipid	48	49
Phospholipid	52	54

The next step was to determine whether or not the sperm could utilize added substrate. It has been shown that mammalian sperm can utilize added substrate. Unfortunately none of the compounds tested, which included a wide variety of carbohydrates and the usual intermediate compounds in carbohydrate and fat breakdown, had any effect on the oxygen uptake of the sperm. Certain materials, such as glucose, fructose, sorbose, etc., were capable of prolonging sperm life by as much as 300 per cent. however. This would indicate that these compounds were able to penetrate only very slowly, and thus that their effect could not be evident in the short time involved in a Warburg determination.

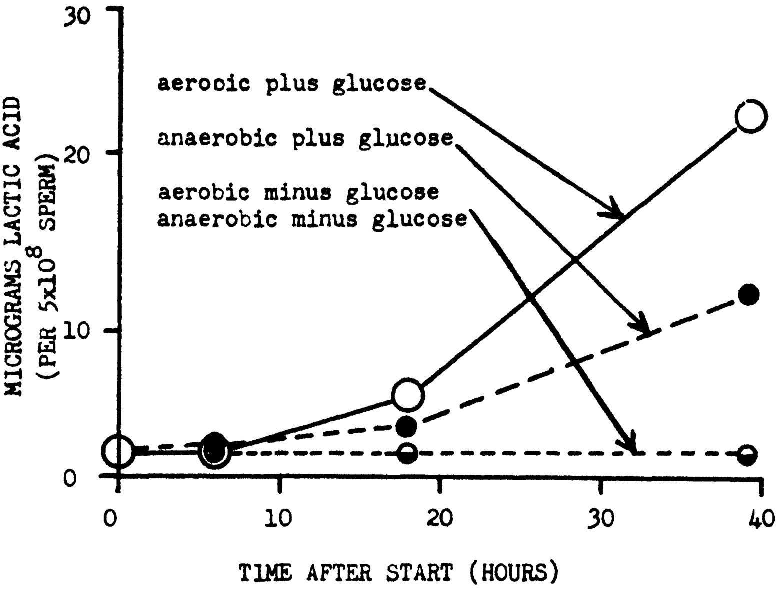

FIG. 1. Lactate production of sea urchin sperm.

The intermediate pathways through which the sperm break down carbohydrate were next investigated. While mammalian sperm appear to be preferentially glycolytic in their metabolism even under aerobic conditions, it was found that sea urchin sperm can produce only small quantities of lactic acid from carbohydrate. Fig. 1 shows the lactate production by sea urchin sperm under various conditions. It will be noted that lactate production is greater under aerobic conditions, and is markedly increased by the addition of glucose. Most tissues show an increase in the rate of glycolysis under anaerobic conditions. None of the substrates tested were capable of maintaining sperm motility under anaerobic conditions. This is in contrast to mammalian sperm, which survive longer under anaerobic conditions than in the presence of oxygen.

Fig. 2 shows the effect of certain inhibitors on the rate of lactate production by sperm under aerobic conditions.

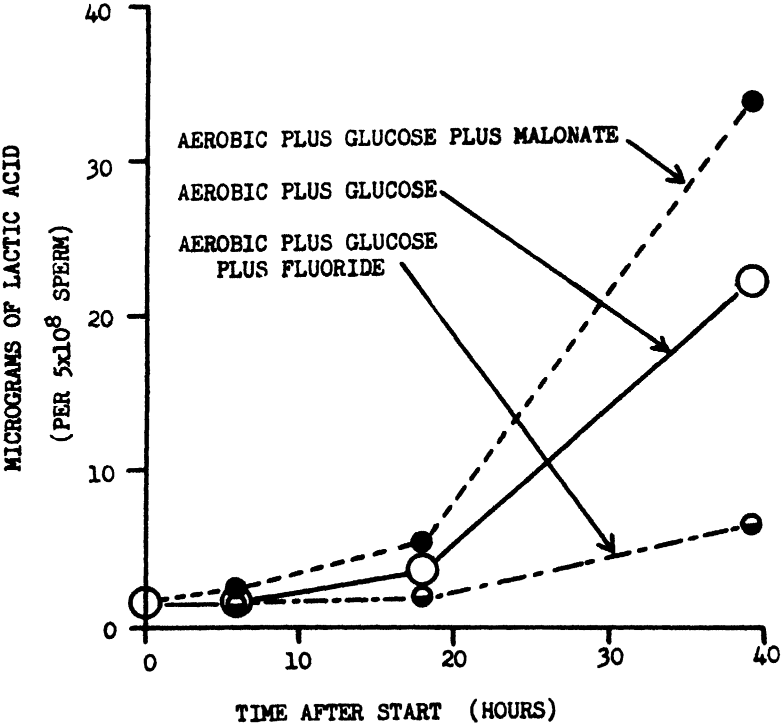

FIG. 2. Lactate production of sea urchin sperm in the presence of certain metabolic inhibitors.

Added fluoride inhibits the formation of lactate in the presence of glucose. Malonate on the other hand increases the rate of formation of lactate.

It was not found possible to demonstrate the presence of the enzyme phosphorylase in a brei of sea urchin sperm. It might be that the system is inactivated in the preparation of the brei. The use of intact sperm gave negative results also, but this would be expected because of the relative impermeability of the sperm. A phenomenon was observed, however, which might be related to the process of phosphorylation. If glycogen or glucose and inorganic phosphate were added to a sperm brei it was observed that the inorganic phosphate concentration of the system rapidly decreased. This might indicate that these materials were being phosphorylated inasmuch as this rapid decrease was not observed in the absence of added glycogen or glucose.

One of the stages in the breakdown of carbohydrate according to the classical schemes of glycolysis is the formation of fructose diphosphate. Fig. 3 shows the formation

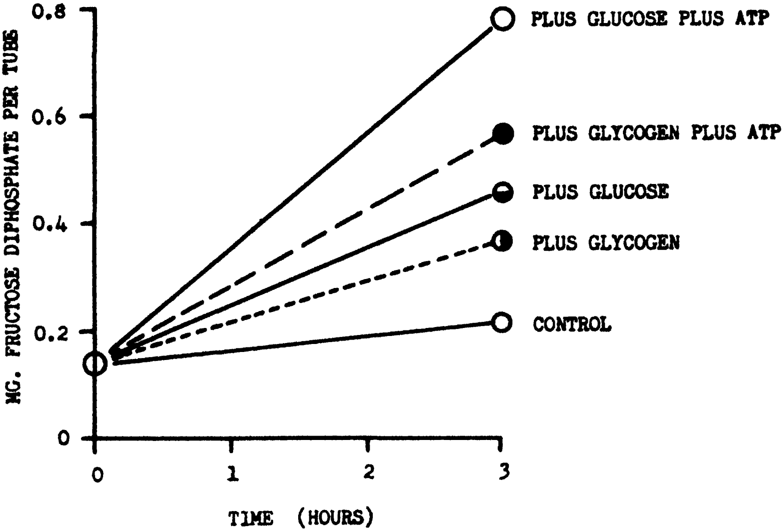

FIG. 3. Formation of fructose diphosphate by sea urchin sperm brei

of this compound under various conditions in a brei of sea urchin sperm. The curves show that the rate of formation of fructose diphosphate is significantly increased by the addition of glycogen or glucose. The rate of formation is increased further by the presence of ATP. These results indicate that the formation of fructose diphosphate in sea urchin sperm from glucose and glycogen probably occurs in the same manner as in yeast, mammalian muscle and most of the other tissues which have been studied.

In the classical schemes of glycolysis, fructose diphosphate is converted to triose phosphate. Fig. 4 shows the conversion of fructose diphosphate to triose phosphate by a brei of sea urchin sperm. Calculations show that this conversion occurs at roughly the same rate as that carried on by the isolated nuclei of rat liver cells, the only tissue comparable to sperm for which data were available.

The formation of pyruvate and lactate from triose phosphate was not observed directly. However, the overall conversion of hexose to lactate, and the intermediate conversion of hexose to fructose diphosphate, of fructose diphosphate to triose phosphate and of pyruvate to lactate have been observed. Thus it would appear that sperm could carry out the conversion of triose phosphate to pyruvate and lactate. Further, it has been shown that the rate of lactate formation from hexose was inhibited by the addition of fluoride. It is known that fluoride somewhat specifically inhibits a reaction in the series of reactions by which triose phosphate

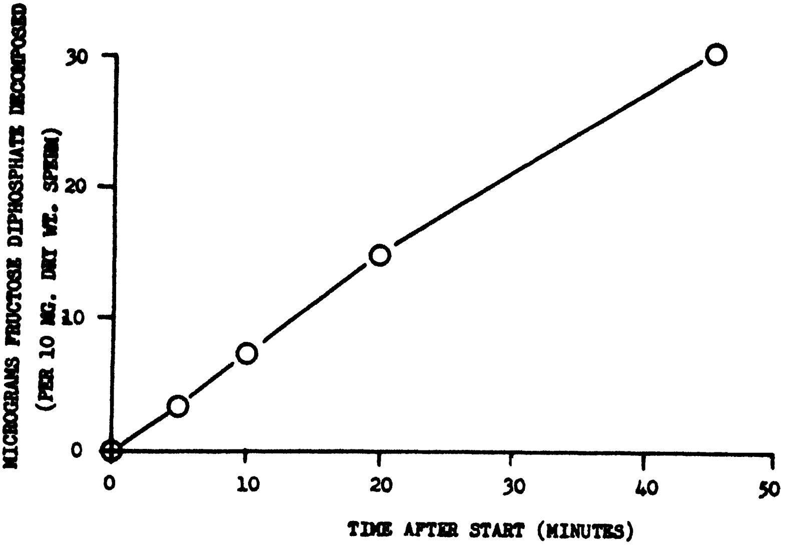

FIG. 4. Aldolase activity of sea urchin sperm brei.

is converted to pyruvate and lactate. It was also observed that the inhibition of the oxygen uptake of sea urchin sperm by added fluoride could not be reversed by the addition of hexose or of any other compounds above 2-phosphoglyceric acid, while the inhibition could be reversed by compounds below 2-phosphoglyceric acid such as pyruvate. Thus it may be concluded that the reactions involved in the conversion of triose phosphate to pyruvate and lactate in sea urchin sperm are essentially the same as those occurring in muscle and in yeast.

The mechanisms involved in the oxidation of three carbon compounds (pyruvate) are not nearly as well understood as are those involved in glycolysis. Probably the best mechanism suggested to date for this process is that of Krebs and his coworkers (1937–1943) which is commonly known as the Krebs tricarboxylic acid cycle. According to this suggestion, pyruvate and oxalacetate are condensed, and the product is oxidized and decarboxylated to yield cis-aconitic acid which adds water to form isocitrate. This is oxidized and decarboxylated to yield α-ketoglutarate which in turn is oxidized and decarboxylated to give succinate. The succinate is oxidized to fumarate, and this is converted to malate which is oxidized to oxalacetate.

Addition of intermediates of the Krebs cycle to sea urchin sperm had no effect on the oxygen uptake of the tissue. Further, sperm were only very slightly inhibited by the addition of malonate, a specific inhibitor of succinic dehydrogenase, which is one of the key enzymes of the Krebs cycle. It would appear then that the sperm are relatively impermeable to these compounds as they are to carbohydrates and to the intermediates of glycolysis.

It has been established that tissues generally are more permeable to unionized materials than to ionized. Thus it might be supposed that esters of the intermediates of the Krebs cycle would penetrate into the sperm, be hydrolyzed there, and then be acted upon by the enzymes involved in the cycle. It was found however, that instead of increasing the rate of oxygen consumption of the sperm, the esters acted as inhibitors. Ethyl citrate, ethyl succinate, ethyl malonate, ethyl fumarate and ethyl malate all gave approximately the same concentration versus inhibition of oxygen uptake curves. This suggested that the esters might be acting as competitive inhibitors of the corresponding salts in a system similar to the Krebs cycle. This possibility was tested for by using in vitro systems of succinic and malic dehydrogenases from the sperm. It was found that the esters acted as highly specific, competitive inhibi-

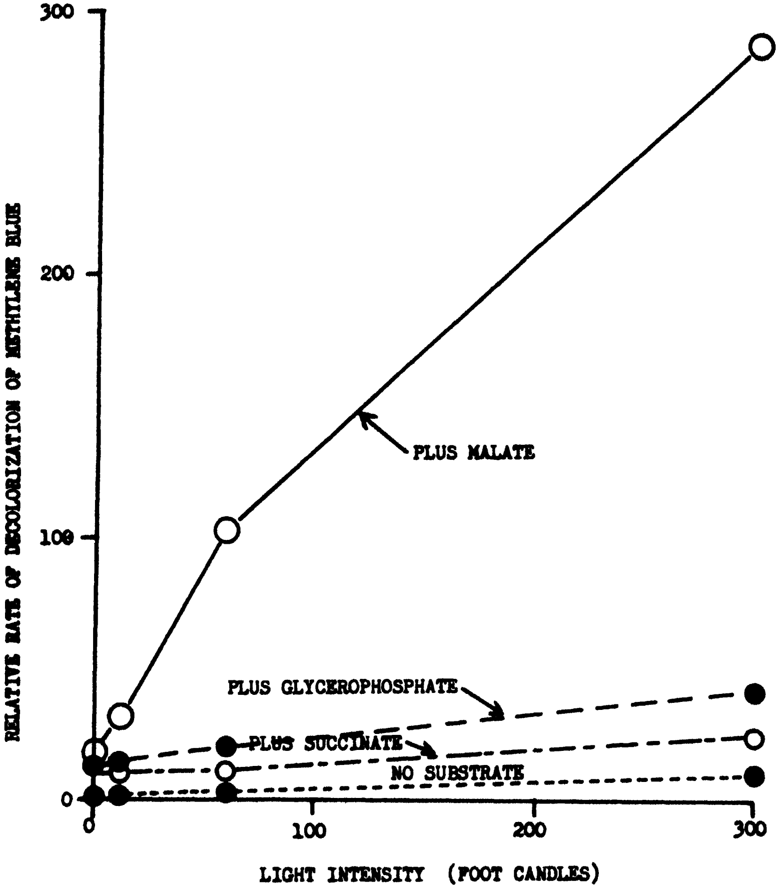

FIG. 5. Effect of white light on the activity of sea urchin sperm dehydrogenases as measured by the Thunberg technique.

tors of the corresponding dehydrogenases. It was also shown that sperm breis could interconvert succinate, fumarate and malate. Thus it would appear from the above indirect evidence that some mechanism resembling the Krebs cycle is in operation in sea urchin sperm.

An interesting phenomenon was observed in connection with the work on dehydrogenases. It was found that the apparent activity of the malic dehydrogenase system was markedly accelerated by exposure to white light. This is shown in Fig. 5. It was found further that this effect was produced only by the red region of the spectrum. This may correlate with an observation made by Professor MacGinitie. He observed a number of years ago that the motility of Urechis sperm is greater in light than in the dark.

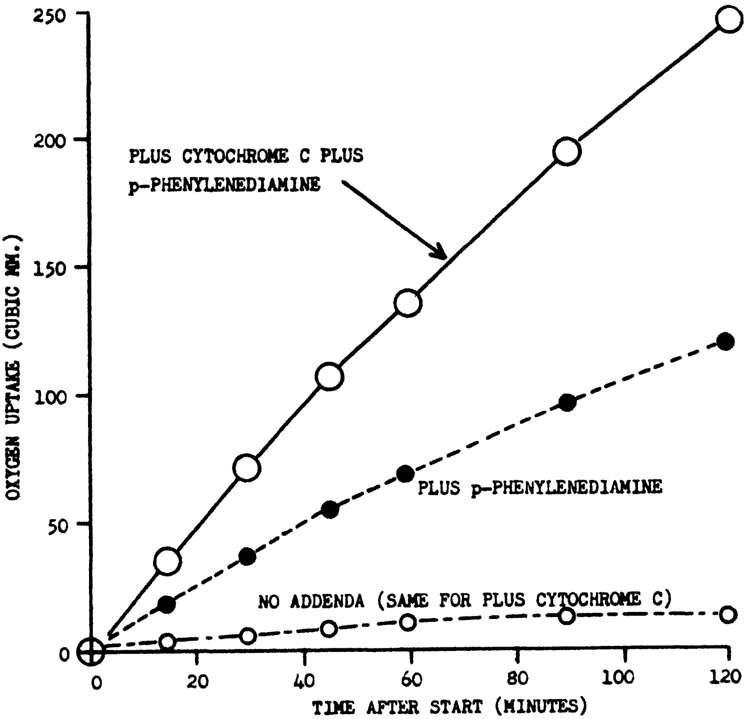

FIG. 6. Cytochrome oxidase activity of sea urchin sperm brei.

The terminal oxidase of mammalian sperm has been shown to be cytochrome oxidase. Sea urchin sperm also contains an active cytochrome oxidase, as is shown in Fig. 6. The curves show that the rate of oxidation of p-phenylenediamine by a sperm brei is markedly accelerated by the addition of cytochrome c. This indicates the presence of cytochrome oxidase in the sperm. Catechol was not oxidized by the sperm.

An adequate explanation of the coupling between the energy produced by metabolism, and the utilization of this energy by cells to perform work has not been described. It would appear that in most tissues energy can be transferred by means of so-called high energy bonds, such as the two terminal phosphate bonds in adenosine triphosphate (ATP). It was found that sea urchin sperm contain relatively large quantities of a barium insoluble, acid labile phosphate ester which might be ATP. Further it was found that the sperm contain a highly specific ATP-ase. This is shown in Table 2.

TABLE 2

Phosphatase Activity of Strongylocentrotus Sperm

(Expressed in terms of relative change in inorganic phosphate concentration over zero time)

Substrate	15 min.	30 min.	60 min.
None	0.4	4.8	9.2
Glucose-1-phosphate	— 2.8	— 2.0	— 3.2
Fructose-1, 6-diphosphate	0	0	3.6
Na glycerophosphate (52% a)	0	1.6	4.0
ATP (sodium salt)	27.2	54.8	80.4

As the data show, sperm brei has a very active ATP-ase, but apparently lacks phosphatases for the other phosphate esters.

In connection with sea urchin sperm it was of interest to observe the effect on sperm metabolism of the material from the surface of the eggs (fertilizin) which specifically agglutinates homologous sperm. It has been reported for some species of sea urchins that fertilizin increases the metabolic rate of the homologous sperm. However, with the species used for this work it was found that fertilizin inhibited the rate of oxygen uptake. It has been suggested that the agglutination per se of the sperm might cause this decrease in rate of oxygen uptake. This possibility could be tested for because, as Tyler (1941) has shown, fertilizin can be converted to a form (univalent) in which it is still capable of specifically combining with the sperm, but can no longer produce agglutination. This can be done, for example, by irradiating the fertilizin with ultraviolet light. Fig. 7 shows that fertilizin in the univalent form, which does not produce agglutination, no longer inhibits the oxygen uptake of the sperm.

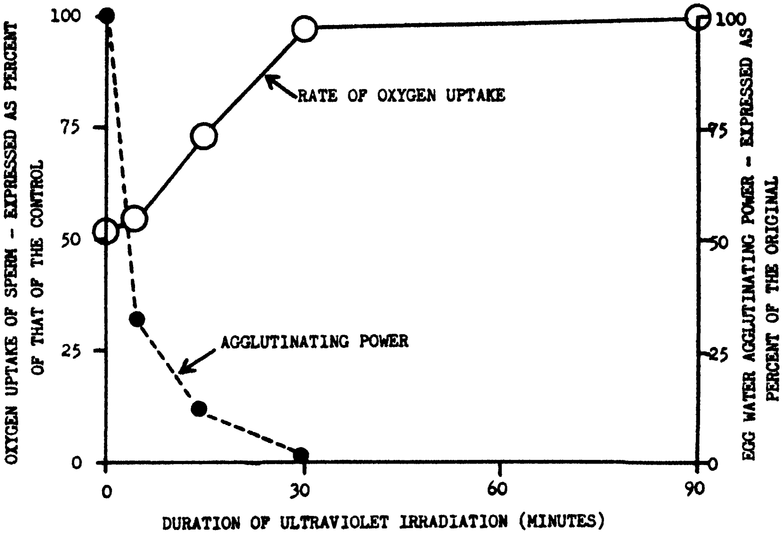

FIG. 7 Effect of fertilizin irradiated with ultraviolet light on the rate of oxygen uptake of homologous sea urchin sperm.

It was of interest to examine the relation between the metabolism of sperm and its ability to fertilize. The fertilizing power of sperm may be determined by making serial dilutions of the sperm in sea water, and adding a known number of eggs to a constant volume of each sperm dilution. The per cent. of eggs fertilized in the various dilutions can be determined, and from these data the concentration of sperm necessary to give 50 per cent. fertilization can be calculated. This figure may be used as an indication of the fertilizing power of a sperm suspension.

As was mentioned earlier, the presence of certain carbohydrates prolongs the life of sea urchin sperm. It can further be shown that the ability to fertilize is maintained for longer periods in the presence of these substrates. In connection with the study of sperm metabolism it was customary to observe the degree of motility and the fertilizing power of the sperm at the end of each Warburg determination. This resulted in three types of data for comparison in each treatment of the sperm: relative oxygen uptake, degree of motility and fertilizing power. The results obtained with a large number of metabolic inhibitors indicated in general that the rate of oxygen uptake of the sperm, the motility and the fertilizing power were directly correlated. Thus it would seem that in these experiments the fertilizing power of a sperm suspension is proportional to its motility (and rate of oxygen uptake). This relationship is apparently found generally. With farm animals, for example, the rate of respiration of the sperm is used as a practical measure of sperm viability and fertilizing capacity.

Motility and concurrent rate of oxygen uptake are not the only criteria of the fertilizing power of a sperm suspension, however. It has been shown by Lillie (1913) and by Tyler (1941) that sea urchin sperm which have been agglutinated by homologous fertilizin show a spontaneous reversal of the agglutination after a time. The reversed sperm appear to be just as motile as before they were agglutinated, but they are incapable of fertilizing eggs. Tyler (1941) showed that if sperm were treated with acid sea water, a treatment which is known to partially remove the specific combining material from the surface of the sperm, the fertilizing capacity of the sperm decreased more rapidly than did the rate of oxygen uptake. Tyler concluded from this that part of the decrease in fertilizing power was due to a removal of the surface material and that this material therefore played an essential rôle in the process of fertilization. Tyler also showed that the specific combining material on the surface of the sperm was protein in nature. This suggested the possibility that the material could be removed by means of a proteolytic enzyme, which treatment should result in a complete loss of fertilizing power without any particular decrease in the motility or rate of oxygen uptake. The results of such an experiment are shown in Fig. 8. Crystalline chymotrypsin was used for the enzyme.

The curves show clearly that the oxygen uptake of the sperm in the enzyme remained almost the same as that of the control, while the fertilizing power of the sperm in the enzyme fell to zero after a rather short time. It was also observed that the degree of agglutination of the sperm with added fertilizin fell off at about the same rate as the fertilizing power. It would appear, then, that the fertilizing power of sea urchin sperm is a function of two independent factors, (1) the degree of motility of the sperm, which is correlated with its respiratory activity, and (2) the presence of the specific combining substance in a reactive condition on the surface of the sperm.

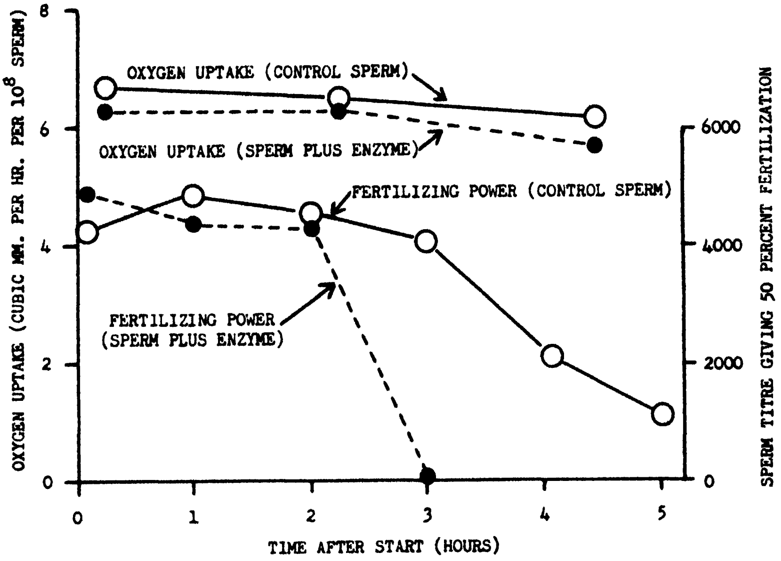

FIG. 8. Effect of crystalline chymotrypsin on the oxygen uptake and fertilizing power of sea urchin sperm.

LITERATURE CITED

Krebs, H. A.
1943. *Adv. in Enzymology,* 3: 191–252.
Lardy, H. R., and P. H. Phillips
1941. *Am. Jour. Physiol.,* 134: 542–548.
Lillie, F. R.
1913. *Jour. Exp. Zool.,* 14: 515–574.
Mann, T.
1946. *Biochem. Jour.,* 40: 481–491.
Tyler, A.
1941. *Biol. Bull.,* 81: 190–204.

DISCUSSION

CHAIRMAN ENGLE: Thank you, Dr. Spikes. The use of the sperm as an object for metabolic studies is something that has gotten under way slowly, but it seems to be gaining in general interest. In the metabolic study on sperm, species differences are observed. It adds a great deal to our knowledge of the sperm cell to observe these differences. Even though species differences occur, one is impressed with the general pattern of uniformity from sea urchins to man. Is there any general discussion of this most interesting topic?

DR. HOROWITZ: Were these experiments under aerobic conditions done with living sperm or sperm brei?

DR. SPIKES: Living sperm. I was never very successful in getting lactic acid production with sperm brei.

DR. HOROWITZ: Then glucose does get in?

DR. SPIKES: Yes, but very slowly. It apparently doesn't penetrate during the course of an ordinary manometric experiment. If the sperm are in contact with it for 12 to 24 hours some glucose apparently gets in, however.

DR. HOROWITZ: Is the lactic acid oxidized by the sperm?

DR. SPIKES: Apparently not.

DR. PEASE: I wondered if you made any effort at all to use different parts of the sperm in such metabolic studies. I suppose it is the middle piece that you are mostly concerned with.

DR. SPIKES: In bull sperm it has been shown that the cytochrome system is largely localized in the mid-piece and tail. We don't have such data on sea urchin sperm.

DR. PEASE: How about the acrosome? Does it have any appreciable metabolism that you know of in the vertebrate sperm?

DR. SPIKES: There is apparently nothing in the literature on that.

DR. CHAMBERS: Were the phosphatase experiments done with sperm breis?

DR. SPIKES: Yes, very little phosphatase activity was found using intact sperm.

DR. CHAMBERS: Even on ATP?

DR. SPIKES: Yes. There was a slight ATP-ase activity with living sperm, but not as great as with the brei. Apparently the material didn't get in. Dr. Chambers, didn't you find that such materials as radioactive phosphorus penetrated the sperm very slowly?

DR. CHAMBERS: Yes, at about the same rate as into the unfertilized egg.

DR. DELBRUCK: How do you measure motility?

DR. SPIKES: Simply by examining the sperm suspensions under the microscope and making a subjective estimate of their motility. It is possible to classify sperm into about six motility groups by such a method.

CHAIRMAN ENGLE: Is your criteria of motility vibratory action or progression?

DR. SPIKES: It is vibratory action. Motility is a rather difficult point to determine actually. I have never heard of a good method of measuring it.

DR. DELBRUCK: You implied a relationship between motility and oxygen uptake. You had a beautiful curve.

DR. SPIKES: If you were to hand me sperm suspensions I could classify them into six different groups on the basis of motility. I wouldn't care to be any more accurate than that.

DR. DELBRUCK: This enzyme reaction, that was photocatalyzed, it was a dehydrogenation of malic acid?

Dr. Spikes: Yes, as determined by the regular Thunberg method.

Dr. Delbruck: What sort of an enzyme is that?

Dr. Spikes: In most tissues it is a system involving the dehydrogenase, coenzyme 1, and diaphorase. However, the whole system is not known for sea urchin sperm.

Dr. Delbruck: You don't know?

Dr. Spikes: Not in this particular system.

Dr. Delbruck: Is this photocatalytic effect unique?

Dr. Spikes: There is one reference in the literature which indicated that a certain system involving diaphorase and methylene blue was slightly activated by red light. I was never able to see the original paper, however. I thought Dr. Haas might be able to help us out. He has been working with this yellow enzyme system which is affected by light.

Dr. Delbruck: Is the enzyme destroyed by light?

Dr. Spikes: I really don't know. It might be that some coenzyme system or some inhibitor system is affected by the light rather than the dehydrogenase itself. The malic dehydrogenase system is somewhat different from the other dehydrogenases in sperm. If you lyophilize sperm, you can still get a very nice succinic dehydrogenase activity, but the malic dehydrogenase activity is destroyed.

Dr. Delbruck: It is destroyed by what?

Dr. Spikes: Lyophilizing.

Dr. Delbruck: How did you run across this photosensitivity?

Dr. Spikes: I just had a series of tubes set up on the desk, and I noticed that on the side toward the windows the tubes were decolorizing much more rapidly than on the other side.

Dr. Delbruck: And how were these measurements then done?

Dr. Spikes: I didn't have much equipment or time. I simply worked in a dark room and set up tubes at different distances from the light source. I used an exposure meter to determine light intensity and then simply observed the rate of decolorization.

Dr. Delbruck: Decolorization of what?

Dr. Spikes: Methylene blue.

Dr. Tyler: You have to add cyanide in this system.

Dr. Spikes: Oh, yes.

Dr, Delbruck: Is this enzyme well known in the sense that it has been purified or crystallized?

Dr. Spikes: I don't think it has been crystallized.

Mr. MacGinitie: Is there any correlation between the rate of dispersal and motility in the sperm? Would it be possible to put in spots of sperm and determine a better rate of motility by the rate of dispersal?

Dr. Spikes: I spent a lot of time in attempting to devise a good method for objectively determining sperm mobility. That was one of the methods we considered, but it was never tested.

Chairman Engle: If there are no further questions, we will proceed to the remaining paper by our guest from Copenhagen, Dr. Erik Zeuthen, on " Oxygen Consumption during Mitosis; Experiments on Fertilized Eggs of Marine Animals."

OXYGEN CONSUMPTION DURING MITOSIS; EXPERIMENTS ON FERTILIZED EGGS OF MARINE ANIMALS[1]

DR. ERIK ZEUTHEN

Hopkins Marine Station, Pacific Grove, California, and
University of Copenhagen

Immediately after fertilization the respiratory rate of the egg may undergo considerable changes; in some species it drops, and in other species it increases. After these initial adjustments of the respiratory rate (oxygen uptake per unit of volume) are over, it can be shown that the rate of oxygen uptake is very much the same in a great many different eggs (Whitaker, 1933). Now the fertilized egg starts cleaving, and cleavage follows cleavage at even time intervals, or at time intervals which quite gradually become longer and longer as development proceeds. In this way the egg divides into a great many blastomeres, which may become very small, their aggregate volume all the time being equal to that of the unsegmented egg. Within the single embryo, each mitotic cycle cleaves all blastomeres almost simultaneously, and the same is also true when we consider a great number of eggs. They divide simultaneously, provided the eggs are from the same female, that they are fertilized at the same time, and that they are kept at the same temperature.

In the eggs of the frog, *Rana platyrrhina*, the first three to four division cycles have been studied; the same is so for the egg of the worm, *Urechis caupo* (in the latter case the synchronicity of the divisions within the single embryo is known to stop after the fourth division). The echinoderm eggs are very well suited for the studies of which I am going to speak, because the first eight to ten cleavages seem to take place in a beautiful rhythm.

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[1] Presented before the Conference on Problems of General and Cellular Physiology Relating to Fertilization, sponsored by The Committee on Human Reproduction of the National Research Council at the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, January 22, 1949.

It is a characteristic feature of all cleaving eggs that a smooth increase in respiratory rate can be demonstrated during the cleavage period. So, for instance, Lindahl (1939) demonstrated that the respiratory rate of the sea urchin egg increases steadily up to the hatching stage (probably around one thousand cells in the embryo). At about the time when hatching occurs, the respiratory rate remains constant for some time, but another rise in respiratory rate sets in as gastrulation and visible differentiation begin. Up to the stage of hatching, the respiratory rate follows an S-shaped curve. The present paper deals mainly with a fine analysis of this curve in the hope of finding " bumps " corresponding to the mitoses going on. It is important to mention that during this early period the segmentation of the egg into a great many blastomeres appears to be the dominating feature of development. For that reason we dare make the assumption that the gradual change in respiratory rate has some intimate connection with the mitoses going on at the same time.

The first series of experiments (Zeuthen, 1946) to be reported were performed on the frog egg. It was the aim to find out whether the respiratory rate is absolutely uniform or not at the times when this egg undergoes the first, second, and third cleavages. At that time I worked in the Carlsberg Laboratory with Linderstrøm-Lang (1937, 1943) and Holter (1943), who jointly developed the Cartesian diver micro-respirometer. It became apparent, however, that the high sensitivity of the Cartesian diver was not high enough. So the diver was modified to give increased sensitivity for slight respiratory variations. In other respects the diver used was a poor one, but anyway it became evident that the three first mitoses in the frog egg were accompanied by slight respiratory variations (Fig. 2). During the early part of each mitosis there was a slight increase in respiration and the cleavage furrow started digging into the egg during this period (according to Brachet [1934] the

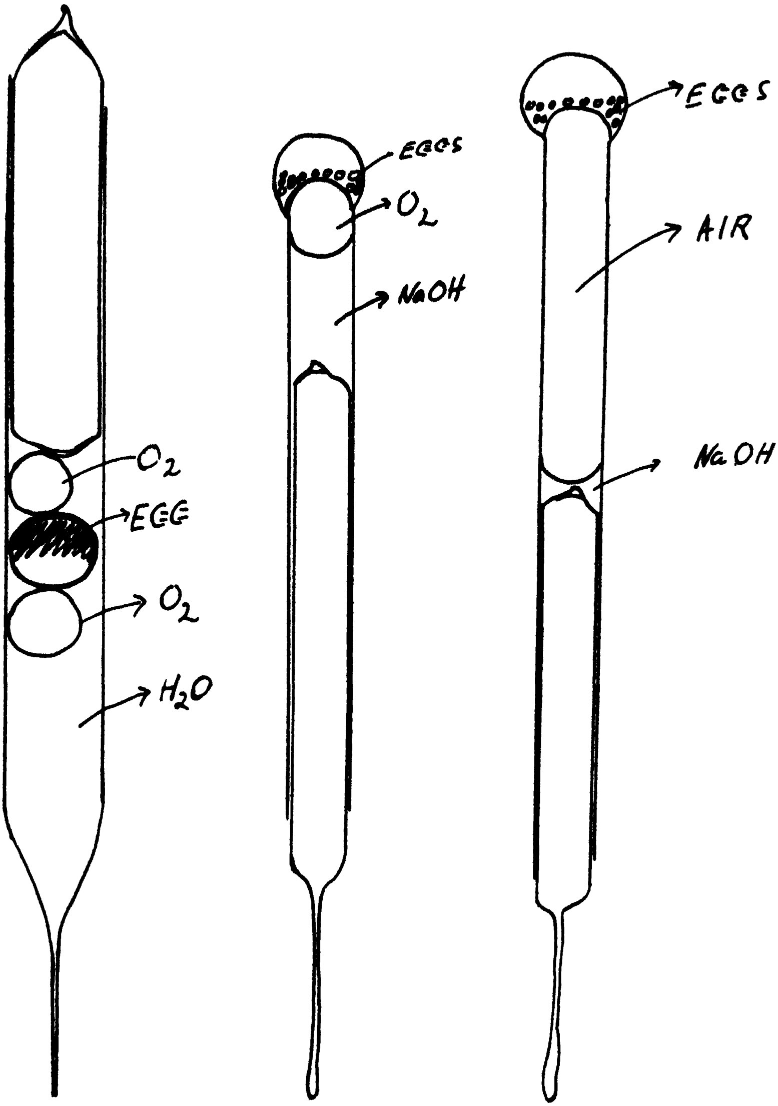

FIG. 1. Three types of Cartesian divers as used for studies on respiration during cell division. *Left:* Diver used in studies on the frog egg (Zeuthen, 1946). The pressure variations in two oxygen bubbles situated close to the egg are followed. No CO_2 absorption, but most of the CO_2 formed is held in solution by the water in the diver. *Middle:* Diver used for studies on the egg of Urechis. *Right:* Diver used in connection with a very sensitive manometer. Used for studies on the eggs of *Strongylocentrotus franciscanus* and *Dendraster excentricus.*

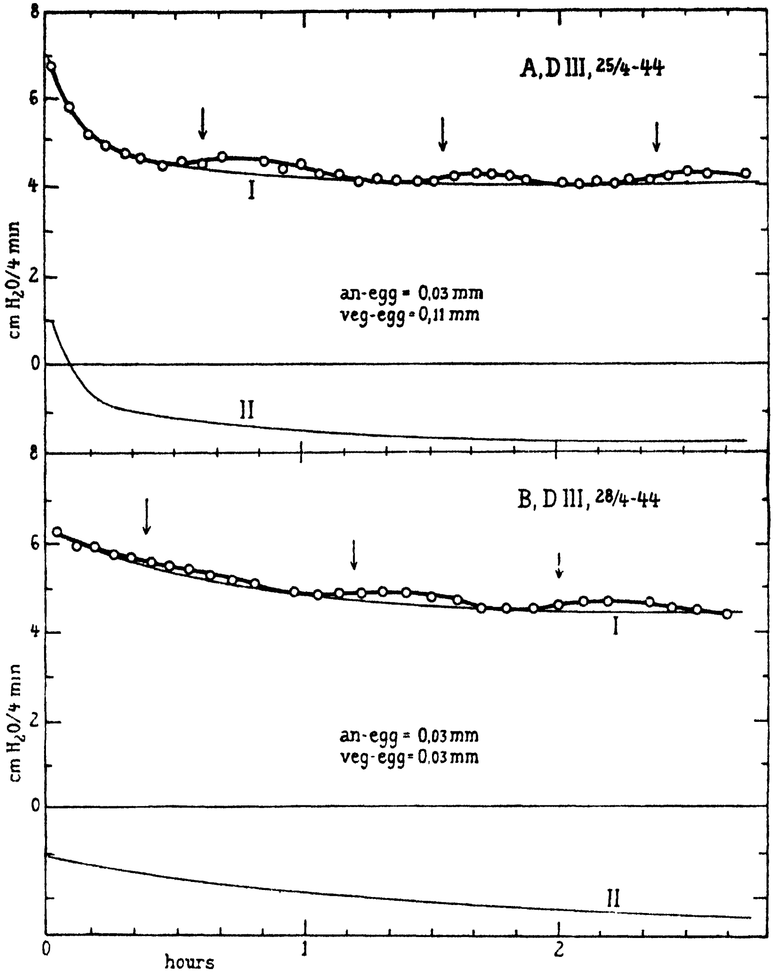

FIG. 2. Two experiments on eggs of the frog, *Rana platyrrhina*. There is one respiratory wave per mitosis. Arrows indicate the time of appearance at the animal pole of the first, second and third division furrows. The initial drop of the curve to the left in the figure is due to technique used (Zeuthen, 1946).

appearance of the cleavage furrow coincides with the prophase). The respiration attains a maximum some ten minutes after the cleavage furrow appears, and after this it starts dropping again. Thus, the respiratory rate fluctuates up and down in the same rhythm as that of the cell divisions going on. The respiratory curve looks like a series of sine waves, one wave per mitosis. Brachet, who used the Fenn technique, had earlier (1934) described another curve for the frog egg having two somewhat larger variations per mitosis. The discrepancies between Brachet's data and mine have never been well explained. As an argument in favor of my results, I mention that I have found essentially constant results in different kinds of eggs (see later).

Linderstrøm-Lang (1946) subjected these results obtained with the diver to a very detailed discussion. The point is that the diver used was specifically adapted for the purpose of detecting a respiratory rhythm, if present at all. This was accomplished at the price of accepting certain drawbacks: In the diver there is no mechanical·stirring so that a diffusion path exists between the metabolic centers in the egg and the recording system (two air bubbles situated next to the egg, comp. Fig. 1, left). Moreover, the separation between O_2 and CO_2 in the diver is incomplete, being based on the different solubilities of the two gases in water. These circumstances led Linderstrøm-Lang to the conclusion that the respiratory variations found were indicative of somewhat larger variations in the egg itself. It was considered, however, that the variations in the egg were of the same type as those recorded with the diver, *i.e.*, sine waves with wave heights about 7–11 per cent. of the average respiratory rate of the egg (the height of the recorded waves were 4.5 per cent.). In subsequent experiments on eggs of marine animals full advantage was taken of Linderstrøm-Lang's results: complete CO_2 absorption was secured, the alkaline seal being separated from the droplet containing the eggs. At the same time the diffusion path between the eggs and the air bubble of the diver was reduced to about one-half mm. Due to both circumstances, the *diver should record the respiratory variations in the eggs without either damping or delay.* The divers adopted are shown in Fig. 1, middle and right. One can see from Fig. 1, middle, right, that the diver floats in such a way that the eggs drop down on the interphase between water and air, where they become densely packed. This reduces the diffusion path in the system to a minimum, fortunately without influencing the development of the eggs. Fig. 3 shows an experiment with several hundred eggs of the worm Urechis. The oxygen uptake of the eggs fluctuates up and down almost like in the frog egg and there is one respiratory wave per mitosis. The cytoplasmic cleavage occurs at the time when the respiration is decreasing, *i.e.*, later than in the frog egg. Cytological studies (by Dr. Irving A. Tittler) indicate that the interphase-prophase coincides with the minimum oxygen uptake. The metaphase-anaphase take place when the respiration is at its height and anaphase-telophase stages are found during the period of decreasing respiration. The difference between the frog egg and other eggs with regard to the appearance of the cytoplasmic cleavage is probably only apparent: In the frog egg the cytoplasm starts dividing during prophase, when the respiration is increasing, but it takes a long time before the furrow embraces the whole egg. In the small marine eggs studied (which all behave like the Urechis egg) the furrow shows up later, when the respiration is again decreasing and the cells are in the telophase. The time correlation between mitotic stage and respiration may well be almost the same in all eggs studied, but the time correlation between these two events and the cytoplasmic division appears to be different in different eggs, depending perhaps on the amount of cytoplasm to be cleaved. If there is much cytoplasm the cleavage takes a long time, and it starts early in the mitotic cycle (frog egg). In Fig. 3 the dotted line indicates the oxygen pressure in the air bubble. The respiration is independent of the oxygen pressure as it drops from 21 per cent. to 6–7 per cent. of an atmosphere. At oxygen pressures lower than this the respiration is no longer independent of the oxygen pressure. Experiments in pure oxygen result in the same type of curve as that of Fig. 3. Apparently, within wide limits the respiratory waves are independent of the oxygen pressure, which, of course, was to be expected. Experiments have also been run with Urechis eggs at different temperatures. As far as I can judge, the wave phenomenon is the same at all temperatures within which normal development is possible (10–24° C.). Compare Fig. 7.

I mentioned that the divisions are synchronized up to a late stage in the echinoderms. This makes these eggs very convenient objects for the present kind of studies. The first echinoderm egg studied was that of the Swedish sea urchin, *Psammechinus miliaris.* In this egg each mitotic cycle is accompanied by an increase in respiration, the curve for the early divisions looking very much the same as that for Urechis. However, it became apparent that the later division cycles (in which many more cells divide at the same time in the embryo than is the case in the earlier divisions) are accompanied by much larger respiratory variations. This is illustrated by the two curves (two different experiments) of Fig. 4. The reader may be confused by the fact that the general trend of this figure is to show a higher

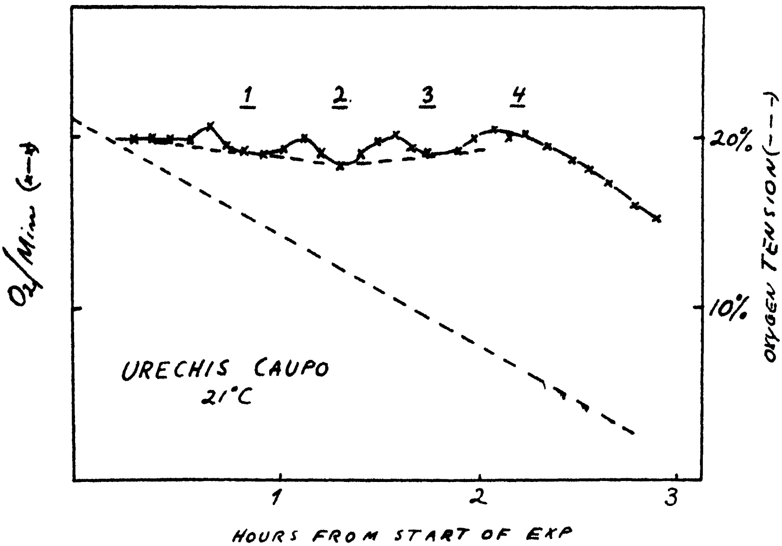

FIG. 3. *Urechis caupo.* Oxygen consumption shows a mitotic rhythm. Times of cytoplasmic division of the four first cell divisions indicated by numbers 1–4. Dotted line shows how the O_2-pressure (per cent. of an atm.) is decreasing during the experiment. At pressures lower than 6 per cent. (in the air bubble) the oxygen tension in the eggs is no longer sufficient to maintain normal respiratory rate. Ordinate in this and following figures are in arbitrary units.

respiratory rate in the first one to two hours of the experiment than might have been expected from the reports of other workers. It is a purely technical deficiency which manifests itself here (also appeared in exp. of Figs. 2 and 7). From the beginning of the experiment the water phase of the diver is always in equilibrium with the atmosphere, but in these experiments the gas in the diver is oxygen. The water therefore takes up oxygen until a new equilibrium has become established within the diver. This results in an oxygen uptake which, of course, must be non-rhythmical and which is superimposed upon the rhythmical respiration of the eggs. In the first one to two hours of the present experiments this apparent respiration constitutes probably 10 to 20 per cent. of the total oxygen uptake measured, *i.e.*, it does not call for a serious revision of the first impression

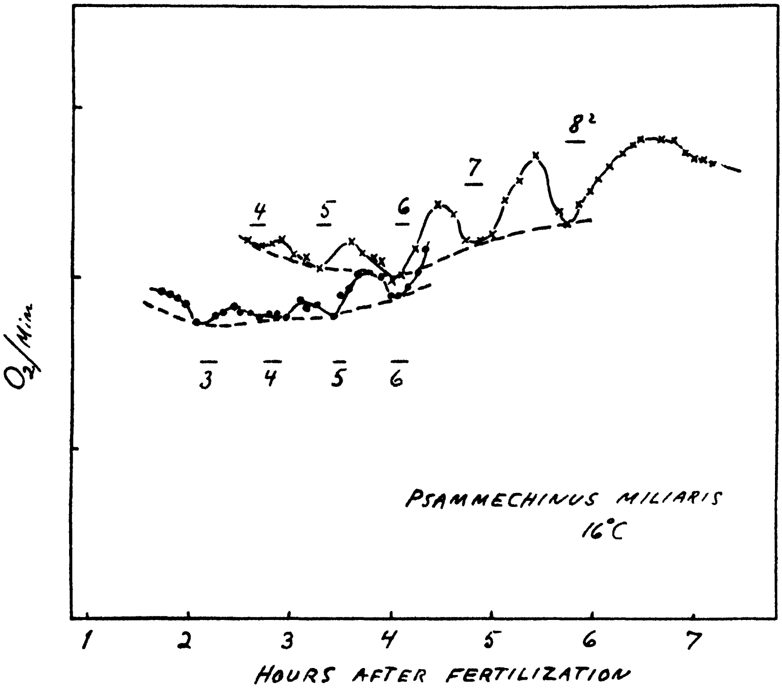

FIG. 4. Two experiments on the Swedish sea urchin, *Psammechinus miliaris*. Cleavage periods of cytoplasm indicated by numbers. Oxygen in divers.

one gets from Fig. 4 of the respiratory rhythm in the Psammechinus egg.

For the experiments on Urechis and Psammechinus a diver looking more or less like the one in Fig. 1, middle, was used. Because the gas phase is small (0.5–1.2 μl) in comparison to the number of eggs present in the diver, we get a very rapid change in equilibrium pressure of the diver (usually around 0.5–1.0–1.5 cm. H_2O/min.). For reasons to be mentioned later, it was desirable to increase the proportion air/gas in the diver by about ten times. This could only be done by increasing the air space of the diver, since the water phase could hardly be very much reduced. Let us consider the following case: The respiratory rate is kept constant. Then a certain increase in gas volume of the diver means a corresponding decrease in sensitivity (*i.e.*, the change of equilibrium pressure/min. gets smaller). But if at the same time we can measure the equilibrium pressure of the diver with a much higher accuracy than before, the sensitivity of the diver as a respirometer may remain unchanged despite the much increased air volume. Fortunately, the Cartesian diver is much more sensitive to pressure changes than thought before. Until recently we have only tried to measure equilibrium pressure to about ± 0.3 mm. H_2O; however, by using a very sensitive manometer of a special design, very small changes in equilibrium pressure can be picked up, the error being about ± 0.03 mm. H_2O. As soon as this became apparent the diver was remodelled from the one shown in Fig. 1, middle, into the one shown in Fig. 1, right. The latter diver, by the way, is much less fragile and therefore much more easy to handle than the former.

Fig. 5 shows an experiment on the eggs of the California sea urchin, *Strongylocentrotus franciscanus.* The experiment runs over 15 hours, all of which are hours of very busy and careful work. For this reason this egg does not lend itself to too much experimentation of this kind. The mitotic rhythm in O_2– uptake, however, seems to be well established. The egg of the Californian sand-dollar, *Dendraster excentricus* (another echinoderm), cleaves twice whenever the Strongylocentrotus egg cleaves once. In the course of seven to eight hours from fertilization it hatches, and eggs in the diver hatch at exactly the same time as do control eggs in the same water-bath, only outside the diver. Fig. 6, upper curve (I), shows that the same respiratory rhythm as in the two other echinoderms can be demonstrated in the Dendraster egg.

I may several times in the above have called the demonstrated rhythm in oxygen uptake a "respiratory rhythm." Strictly speaking, this is not justified. The "respiratory" variations are not so big that it might not be possible to interpret the whole fluctuating curve in terms of a smoothly increasing respiration on top of which a reversible oxidation and reduction of some unknown chemical system is going on, the molecular O_2 being used in this process. This would simulate true variations in respiratory rate. The reason why I have been worrying about this is that Rapkine (1945) claims to have demonstrated a rhythmic (mitotic) variation in the detectable amount of SH– groups in the sea-urchin egg. Before we dare call the demonstrated mitotic fluctuations in oxygen uptake *respiratory* variations, it is required that it be demonstrated that the CO_2 output and the oxygen uptake fluctuate in the same rhythm. Here the reason why I wanted to increase the ratio gas/water in

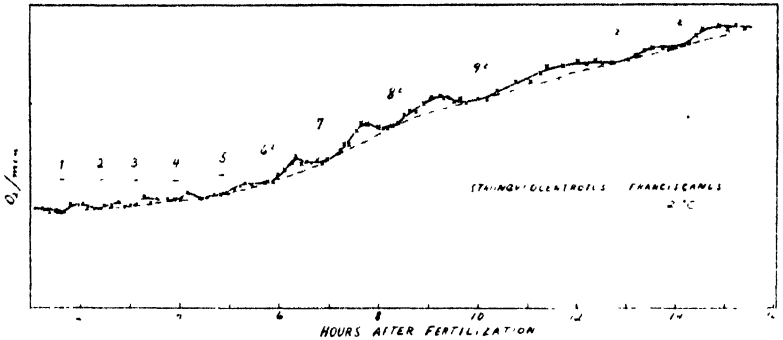

FIG. 5. *Strongylocentrotus franciscanus.* Mitotic rhythm in metabolism. Only the first five divisions could be actually observed in the diver. There are slight indications on the curve that the synchronicity of the divisions in the embryo is stopping after ninth division. Air in diver.

the diver comes in: In the diver of Fig. 1, middle, a very significant part (65 per cent.) of the carbon dioxide will remain in solution in the water even if we do not absorb CO_2 in the neck seal and even if the water in the bottom droplet is slightly acid. In the diver shown in Fig. 1, right, the ratio gas/water is about 5, as compared to about 0.5 in the diver of Fig. 1, middle. It is easy to calculate that if the diver in Fig. 1, right, is tight to CO_2 (which it probably is) and at 20° C. (the temperature is not very significant)

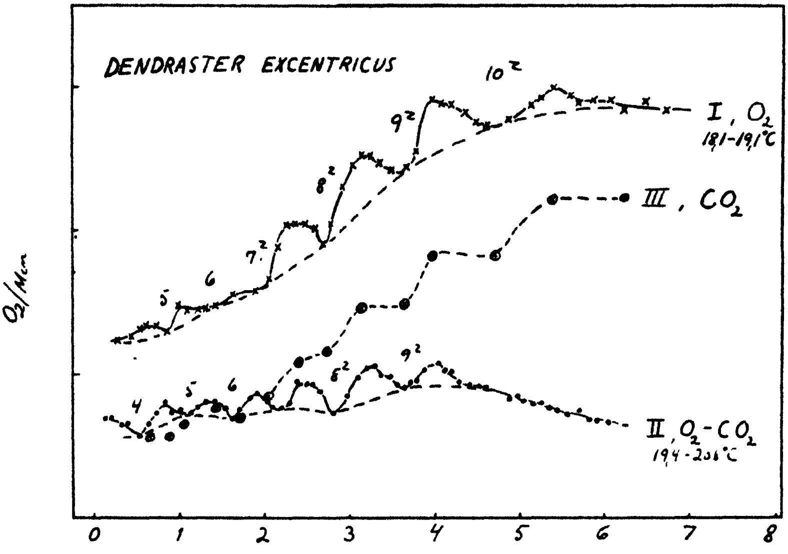

Fig. 6. *Dendraster excentricus.* Mitotic rhythm in oxygen uptake and in CO_2-output. Air in diver. Further explanation in text.

85 per cent. of the respiratory CO_2 will remain in the gas phase and therefore be manometrically measurable as the difference between the rates found with and without CO_2-absorption in comparable experiments.

Whereas the upper curve in Fig. 6 shows the variations in oxygen uptake in the Dendraster egg, the lower curve (II) shows the rate of pressure change when at the same time oxygen is consumed from and carbon dioxide is put out into the gas phase. By subtraction of the two curves we find the CO_2- production (curve III). This curve is interpreted as indicating that the carbon dioxide production follows the same rhythm as does the oxygen uptake; in other words, *the rhythm found is a respiratory rhythm and therefore indicates a rhythmic energy output of the eggs.* Accordingly, these experiments give no evidence for assuming the presence of a reversible oxido-reduction process as proposed above. Admittedly the suggested rhythm in CO_2– output is not by any means as safe as is the statement that the oxygen uptake fluctuates in a mitotic rhythm. Three experiments have been run with carbon dioxide absorption in the diver, all giving identical results for the rhythm in O_2– uptake. Four experiments have been made in which the carbon dioxide was not absorbed. Two of these latter experiments were made in ordinary sea water, the other two were run in artificial bicarbonate free sea water. I could find no difference in the results in sea water and in artificial sea water. By subtracting each of the four experiments without CO_2– absorption from each of the three experiments with CO_2– absorption (12 subtractions) I invariably find that the resulting curve for CO_2– production shows a rhythm (as in Curve III) comparable to that demonstrated for the oxygen uptake. The carbon dioxide rhythm, however, can only be demonstrated for the large respiratory waves of the later division stages. A few words should be said also about the way the curves are subtracted, such as to give the CO_2– production: Seven different experiments, all on eggs from different females of Dendraster, are compared. This can only be done if we accept certain individual differences which exist on the curves. I have made the assumption, however, that curve maxima may safely be subtracted from curve maxima and curve minima from curve minima, provided, of course, that in each case mitoses having the same numbers are compared. This is what I did: I plotted the curves below one another and measured the *vertical* distance between corresponding maxima and minima. This vertical distance was plotted against time. The resultant curve of one such subtraction is the one shown in Fig. 6, III. No correction has been made for the amount of CO_2 (about 15 per cent. of the total CO_2) which must have remained in solution in the water of the diver.

I certainly do not pretend definitely to have solved the CO_2- part of the problem discussed, but I hope to have presented a preliminary attack on a difficult problem, and I

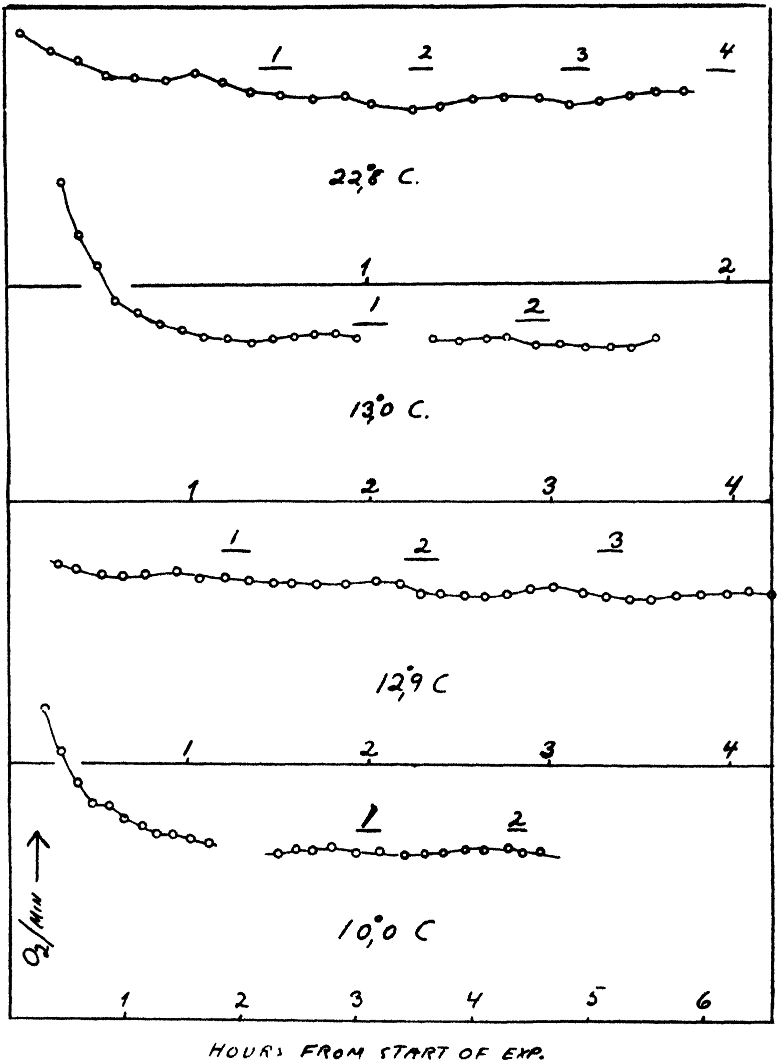

FIG. 7. *Urechis caupo.* Experiments at different temperatures. Slopes to the left in the figure due only to technique. Curves plotted in such a way that they can be directly compared. Wave phenomenon seems to be unaffected by temperature. All experiments with oxygen in the diver.

believe that the conclusion made is the best one to be drawn on the basis of the evidence available.

Next I would like to discuss a little more the shape of the respiration curve found in the five different eggs. I have not mentioned before that only for the first 4–5–6 divisions do we actually know that one mitotic cycle is represented by one respiratory wave. This is so because usually these first divisions—and only these—could be observed in the diver itself. With regard to later stages we are limited to mere guessing, but until we know better it is reasonable to assume that the larger respiratory waves found in all cases correspond to a mitotic cycle. If this is so, we must also assume (comp. the curves) that at the time when the echinoderms are hatching, the mitotic activity is slowed down extremely (by the way, hatching itself does not seem to be accompanied by any change in respiratory rate). As long as in an egg all blastomeres take part in each mitotic cycle, the number of blastomeres in the embryo must increase exponentially with the number of mitotic cycles. At the same time, the total volume of the embryo remains constant. Growth at the expense of outside material does not take place until much later. If we interpret our curves as indicating that each mitotic cycle is accompanied by an extra respiration (equal to the area between the sine curve and a smooth auxiliary curve which touches all valleys of the respiration curve), the question arises: Is the extra respiration a function of the total mass of the embryo or is it a function of the number of cells dividing per mitotic cycle. The answer is that most likely the extra respiration is the same for each early mitotic cycle, but only up to a certain stage (division No. 4–5 in Psammechinus, division No. 6–7 in Dendraster and in Strongylocentrotus, limit unknown for the frog and for Urechis because in these two eggs only the early divisions were studied). Up to this stage, therefore, the extra respiration is a function of the constant mass of the embryo rather than of the rapidly increasing number of cells dividing, and it is of the order of 2 per cent. of the total metabolism only. But after this stage has been passed, the extra respiration becomes more closely a function of the number of cells dividing than of the mass of the embryo. In the first experiments with Psammechinus there was actually during these later stages a doubling of the extra respiration for each new mitotic cycle, the doubling beginning at the 5th division and ending after the 8th division. In other echinoderm eggs this proportionality between extra respiration and number of cell divisions taking place in the latter part of the segmentation period was not nearly so pronounced, but the principle of a larger and larger extra oxygen uptake for each new mitosis is the same (comp. the figures). The conclusion is drawn that a *physiological difference exists between the first 4–5–6 mitotic cycles and the later mitoses, at least in the echinoderm eggs considered, and perhaps more generally, too.* The later mitotic cycles are accompanied by extra respirations which may be, for instance, 10 per cent. of the total metabolism.

The experiments seem to indicate that each mitotic cycle in fertilized eggs is accompanied by an extra respiration, in other words, an extra energy output. In physiology we always like to find an extra work done whenever we find an extra energy output. With regard to the cell division we are utterly confused as soon as we try to define which work is being done. In one experiment on a fertilized frog egg, quite normal variations in oxygen uptake were found; probably the nuclei divided, but the cytoplasm did not. In another egg from the same batch which behaved the same way, the nuclei divided all right. Logically, we can not from this experiment deduce that the respiratory variations are not normally used for cleaving the cytoplasm. The only thing we can say is that the two processes are dissociable, and perhaps they have no connection at all. The fact that in the marine eggs studied the cytoplasm divides at a time when the respiration is already decreasing is a much better indication that the extra oxygen uptake is not used for " cleavage work," a concept which we must remember has never been physically defined.

One would much rather try to find some correlation between extra energy metabolism of the mitosis and some kind of chemical work (spindle formation, other syntheses) which may well take place in the cytoplasm and at the same time be governed by the nuclei or at least timed in the same way as the mitotic cycle of the nuclei. So far, I have been unable, however, in the literature to find any chemical constituent of fertilized eggs, the growth of which could be said to follow a curve that can be correlated with the findings reported in this paper, or rather which can be correlated with the present findings as I have interpreted them. It may be important, however, that the overall metabolism of the eggs starts to rise more steeply at the time when each new mitotic cycle becomes associated with larger and larger extra oxygen uptakes. So the present status of this investigation seems to be that we have found evidence of an extra energy output during the mitosis, but so far we do not know what the cells do with this energy. To throw more light on this problem shall be the object for further studies.

LITERATURE CITED

Borei, Hans
1948. *Arkiv. f. Zoologi,* 40A, Nr. 13.
Brachet, J.
1934. Arch. de Biol., 46: 1.
Conklin, E. G.
1935. Quoted from E. Fauré-Fremiet, "La Cinétique du Developpement," Paris.
Godlewski, E.
1935. Quoted from E. Fauré-Fremiet, "La Cinétique du Developpement," Paris.
Holter, Heinz
1943. Compt. rend. Lab., Carlsberg, Sér. Chim., 24: 399
Lindahl. P. E.
1939. *Z. Vergl. Physiol.,* 27: 136.
Linderstrøm-Lang, K.
1937. *Nature,* 140: 108.
1943. Compt. rend. Lab., Carlsberg, Sér. Chim., 24: 333
1946. Compt. rend. Lab., Carlsberg, Sér. Chim., 25: 229.
Rapkine, L.
1945. Work reviewed in Brachet, J., "Embryologie Chimique," Paris.
Whitaker, D. M.
1933. *Jour. Gen. Physiol.,* 16: 475.
Zeuthen, E.
1946. Compt. rend. Lab., Carlsberg, Sér. Chim., 25: 191

DISCUSSION

CHAIRMAN ENGLE: Thank you very much. The paper is open to discussion.

DR. MACGINITIE: Isn't it possible that the manufacture of some chemical substance such as, for instance, chromatin, would correlate nicely with your results?

DR. ZEUTHEN: I have considered, that since up to the stage of the sixth division (Dendraster) the extra oxygen consumption per mitotic cycle appears to be relatively constant, one must expect a substance which is formed by this extra respiration to grow with a constant rate up to this stage. After this, the extra respiration per mitotic cycle becomes bigger and bigger. So now the hypothetical substance should grow faster and faster. Little is known about the growth of chromatin in these early stages,[2] but something is known about the growth of the aggregate volume of the nuclei in the embryo (Conklin, 1935; Godlewski, 1935). Apparently this volume grows fast in the beginning, but after just about the sixth division (somewhat different in different eggs) the curve flattens out. After this, each nucleus tends to halve its volume at each division, so that the aggregate nuclear volume from now on constitutes an approximately constant fraction of the embryo. These facts do not seem to indicate a close relation between the formation of "chromatin" and the respiratory rhythm which we discuss. Admittedly, however, the aggregate nuclear volume may *quite possibly not be a good measure of anything important.*

DR. HOROWITZ: In Urechis the first thing which happens after fertilization is that the polar bodies appear. Is this connected with any change in respiratory rate?

DR. ZEUTHEN: As far as my experiments go there are two small peaks showing up on the curve at that time. The peaks have about the same height as those accompanying mitosis, but they are shorter in time.

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[2] The recent paper by Villee *et al.* (*Jour. Cell. Comp. Physiol.*, June, 1949) shows that DNA in the Arbacia egg grows not too differently from this hypothetical substance. This changes my answer to Dr. MacGinitie. My results may perhaps correlate with the growth of "chromatin."

DR. TYLER: You didn't mention any experiments on unfertilized eggs, but I presume that they didn't show the peaks.

DR. ZEUTHEN: Experiments on unfertilized eggs of the frog and of Urechis have failed to show any peaks at all. In the frog, I find a quite gradual decrease in respiration, but in Urechis the respiratory rate of the unfertilized egg shows a gradual, very smooth increase.

DR. CHAMBERS: Is there any way of measuring the change in respiration immediately after fertilization with this method? Say, during the first 5, 10 or 15 minutes after fertilization?

DR. ZEUTHEN: Dr. Hans Borei (1948) in Sweden has recently published studies of this kind with the Cartesian diver. The principle is the same as that used for determining respiratory quotients (Needham *et al.*, Holter). By applying excess pressure on the diver for a very short time, a droplet containing eggs is forced down so as to come in contact with another droplet containing an activating agent. Some few minutes, however, may have to elapse before the diver " recovers " from this fairly rough treatment.

CHAIRMAN ENGLE: Are techniques available for a study of nucleic acid which could be compared with your curves?

DR. ZEUTHEN: No studies are available which permit a direct comparison of the thymo- or ribonucleic acid during early development with my curves. People have never bothered with sampling close enough for this.

DR. DELBRÜCK: It seems to me, as I understand it, that these extra oxygen uptakes are not correlated with the formation of new chromosome material. In that case, you would expect each successive bump to be twice larger than the preceding one, and evidence is strong that that is not so. Therefore, whatever the extra oxygen does, it is not directly correlated with the growth of nuclear components.

DR. ZEUTHEN: Only in one case (divisions 5–8 incl. in Dendraster) was each successive bump found to be twice the preceding one. The extra oxygen consumption accompanying earlier divisions (1–about 5–6) appears in all eggs to be independent of the number of cells dividing in the embryo.

The general picture does not seem to give a good correlation with the growth of nuclear components, so far as this growth is known to us.[3]

Dr. Tyler: Concerning Dr. Delbruck's question: The total nuclear volume of the cleaving egg does not double at each division according to the figures of several investigators. It increases slightly.

Dr. Zeuthen: The nuclear volume increases very slightly at that time of development, when I find approximately a doubling of the extra oxygen taken up per mitotic cycle. In earlier division stages when the extra oxygen is approximately constant from one mitotic cycle to the next, the total nuclear volume grows faster, but even during this period there may only in a very few cases be a doubling of the nuclear volume per cleavage. Conklin's curves differ somewhat from species to species.

Dr. Giese: I would like to hear how you compared those experiments on Urechis which were performed at widely different temperatures.

Dr. Zeuthen: Of course, everything goes much faster at high temperature (22°–24° C.) than at low temperature (9°–10° C.); but if the curves obtained at different temperatures are plotted in such a way that they go as closely as possible on top of one another, I can see no certain difference between them. Differences may be present, of course, but in that case they are within the experimental error (comp. Fig. 7).

Dr. Tyler: One curve showed a falling off of the rate of respiration when the oxygen tension dropped below 6 per cent. As I recall the data of Amberson, Tang and Gerard, there is no drop in the rate of oxygen uptake down to very low oxygen tensions.

Dr. Zeuthen: In the experiment with Urechis eggs, the respiratory rate started dropping when the oxygen pressure in the air bubble went below 6 per cent. What is important in this connection is, of course, the oxygen pressure on those eggs which are most remote from the air bubble. This is probably much lower and perhaps close to the figure Dr. Tyler remembers.

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[3] Compare note, p. 319.

Dr. Delbrück: I heard you say, I think, that the time delay in diffusion is negligible. How big is it actually?

Dr. Zeuthen: In the frog egg the mitoses follow one another with a period of around one hour, perhaps slightly less. For this egg Linderstrøm-Lang (1946) feels justified in fixing the limits for the possible delay to 0–7 minutes. The delay for O_2 and for CO_2 is not very different. Linderstrøm-Lang has not yet seen the results obtained for other eggs, but if I understand his mathematics rightly, the delay should in these experiments never exceed two minutes (period of respiration rhythm always larger than 25 minutes, comp. figures).

Dr. Pease: I wonder if this lag is so serious that for this reason you would not be able to identify, for instance, the rise in oxygen uptake with the breakdown of the nuclear membrane. Do you consider that the dissolution of the nuclear membrane initiates the rise in respiration?

Dr. Zeuthen: I do not think that the delay is very important. For the evaluation of the experiments it is probably much worse that the scattering of the observed points makes it impossible to make a very accurate curve drawing. For this reason, I do not find it possible to answer the last point in your question affirmatively. The only thing I can say is that the first part of the mitosis is accompanied by a steady rise in metabolism. After metaphase-anaphase the respiratory rate again gradually decreases.

Chairman Engle: I have a feeling that this respiration is going to continue to rise and fall during the dinner and evening session. I think we will hold those stimulating data and bring this meeting to a close. I want to express from the standpoint of the National Research Council great appreciation for the care Dr. Tyler has taken in making arrangements for this meeting and for the participation of all you learned gentlemen, and especially to the Department of Biology at the California Institute of Technology, our gracious host.

Whereupon the meeting adjourned at 5:00 P.M.

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